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ELECTROPHORETIC STUDIES OF VIRUS-RED CELL INTERACTION: MOBILITY GRADIENT OF CELLS TREATED WITH VIRUSES OF THE INFLUENZA GROUP AND THE RECEPTOR-DESTROYING ENZYME OF *V. cholerae*.

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HANIG (1948) has shown that following treatment of human erythrocytes with a purified preparation of influenza A (PR8) virus, the electrophoretic mobility of the cells decreased from a value of 1·32 to 0·5–0 6 μ/sec./volt/cm. This decrease in the mobility was found to follow the adsorption and elution patterns of the virus and to be dependent upon virus concentration, suggesting that the change was a direct result of the enzymic activity of the virus. As Burnet, McCrea and Stone (1946) had previously shown that cells treated with one strain of virus, though no longer agglutinable by homologous virus, could be agglutinated by certain other strains, it appeared probable that the varying extent of interaction of these viruses with human erythrocytes would be reflected in corresponding changes in the mobility of the cells.

The electrophoretic mobility of human erythrocytes treated with influenza A and B, mumps and Newcastle disease viruses and the receptor-destroying enzyme of *V. cholerae*, have been measured, and the preliminary results published in brief (Ada and Stone, 1950). In this paper, the procedures and results are given in detail.

MATERIALS AND METHODS.

Normal saline.

0·90 per cent sodium chloride in distilled water.

Calcium acetate saline.

12·3 g. sodium acetate, 1·3 ml. 2N acetic acid, 5·0 g. sodium chloride, 1·0 g. calcium chloride, made up to 1 litre with distilled water. pH was 6·2.

Red blood cells.

Human group O cells from a single donor were used. After collection in the presence of sodium citrate, they were washed three times with normal saline and stored as packed cells at 4° C. When used they were never more than 4 days old.

Strains of virus.

Mumps.—Obtained through the courtesy of Dr. J. F. Enders, 1946.
NDV.—Newcastle disease virus of fowls, Victorian strain of Albiston and Gorrie (1942).
MEL.—" Melbourne " strain of influenza A (Burnet, 1935).
PR8.—Influenza A (Francis, 1934).
BEL.—Influenza A, isolated from Victorian epidemic (Burnet, Beveridge, Bull and Clark, 1942).
LEE.—Influenza B (Francis, 1940)
SW.—Shope's strain 15 of swine influenza (Shope, 1931).
MIL.—Influenza B, isolated from Melbourne epidemic (Burnet, Stone and Anderson, 1946).

Stocks were grown in the allantoic cavity of fertile hen's eggs. Mumps virus was inoculated into 8-day eggs and the allantoic fluid harvested after incubation for 5 days. All other strains were inoculated into 11-day eggs, the fluid being harvested after 42 hours' incubation.

Virus eluates.

Packed human red cells were added to virus-containing allantoic fluid at 0° C. to give a 5 per cent concentration. After 30 minutes at 0° C. the mixture was centrifuged (0° C.), the cells washed once with chilled normal saline and resuspended in a volume of saline equivalent to half the volume of the original allantoic fluid. After incubation for 6 hours at 37° C. the mixture was centrifuged and the supernatant fluid collected.

Methanol-precipitated virus.

This was prepared according to the technique of Cox, Van der Scheer, Alston and Bohnel (1947).

Agglutinating dose of virus.

One agglutinating dose was the amount capable of causing partial (+) agglutination of an equal volume (0·25 ml.) of a 1 per cent suspension of normal red cells (human or fowl, as stated) at room temperature, the degree of agglutination being read from the pattern of sedimented cells. The haemagglutinin titre refers to the dilution of virus which caused "+" agglutination under the same conditions.

Antisera.

Immune ferret or rabbit sera were prepared by intranasal or intravenous inoculation, respectively.

RDE (the " receptor-destroying enzyme " of *V. cholerae*).

Crude extract of the 4Z strain was obtained following the method of Burnet and Stone (1947). The enzyme was concentrated by precipitation in the cold

with methanol, purified by absorption and elution from red cells and then further concentrated by precipitation with ammonium sulphate. Full details of the procedure will be published at a later date. The RDE preparation used in all the experiments was a colourless transparent solution with a titre of $2{\cdot}5 \times 10^5$ as determined by the method of Burnet and Stone (1947). One ml. of aliquots of this were diluted 25 times and kept at $-10°$ C. until required.

For use in the treatment of red cells further dilutions of RDE were made in calcium acetate saline.

Stabilization of virus-treated red cells.

Twofold dilutions of antiserum in 0·25 ml. volumes of normal saline were mixed with 0·25 ml. of a 1 per cent suspension of the virus-treated cells. After thorough shaking the cells were allowed to settle at room temperature. The smallest amount of serum inhibiting agglutination was determined by observing the pattern of the sedimented cells and serum added in the same relative amount to the treated cells (in 5 per cent suspension). After standing for 20 minutes at room temperature, the mixture was centrifuged and the cells washed once with normal saline.

Test of red cells for agglutinability.

It was essential that the cells should first be stable under the conditions of testing, i.e. give no agglutination when 0·25 ml. of a 1 per cent suspension was mixed with an equal volume of normal saline and allowed to settle at room temperature. Of a 1 per cent suspension 0·25 ml. volumes were mixed with equal volumes of dilutions of the standard series of viruses containing 5 agglutinating doses for 1 per cent normal human red cells. The degree of agglutination in each tube was read by the pattern of red cells after settling at room temperature.

Microelectrophoresis.

This was carried out in a M/15 sodium potassium phosphate buffer, pH 7·35 $\pm$ 0·05 (Clark, 1920). Cells to be examined were washed once in the buffer before resuspension for measurement. This buffer is not strictly isotonic and cells which have had long treatment with virus, though they remain intact in physiological saline, often haemolyse in this medium. In later experiments this was overcome by the addition of 1 per cent glucose (w/v) to the buffer. This addition did not significantly alter the mobility of the cells. Measurements were carried out in a microelectrophoresis apparatus designed and largely constructed by Mr. H. F. Holden, to whom we owe our thanks. The basic measurements of the cell itself were within the range given by Abramson, Moyer and Gorin (1942). In preliminary measurements, readings at the two theoretical levels were often widely different. After coating the cell before each series of measurements with a 1 per cent (w/v) solution of gelatine in water (Miller, Lauffer and Stanley, 1944), the depth-velocity parabola became symmetrical, and measurements made at the two theoretical levels were usually similar. In practice a minimum of ten readings were made in a coated cell at both levels and the results averaged. After estimation of each sample, the cell was thoroughly washed through with buffer before the introduction of the next sample. Control

estimations involving normal cells were carried out before and after each set of tests. The cell was held in a small bath through which was circulated water at 25 ± 1° C.

EXPERIMENTS.

Treatment of Red Cells with Individual Viruses and RDE.

Red cells were exposed to the action of virus in the form of infected allantoic fluid for a period of several hours at 37° C. Preliminary experiments were carried out with each strain to determine the amount of virus and the time of treatment required to allow the reaction to proceed to equilibrium as judged by changes in electrophoretic mobility and agglutinability with virus.

Following the action of each virus except mumps and NDV, the cells were stabilized by removing residual virus with the corresponding antiserum. This treatment was carried out according to the method described by Burnet, McCrea and Stone (1946), the minimum amount of serum necessary to stabilize the cell suspension, as determined by titration, being used. The technique is given in detail under "Methods." It was essential to use sera in which there was a sufficiently wide zone between the titre of anti-haemagglutinin and T-agglutinin, as the T-agglutinin causes agglutination of virus-treated red cells (Burnet, McCrea and Stone, 1946). If a serum of low anti-haemagglutinin titre only was available it was first absorbed with RDE-treated red cells to reduce the titre of T-agglutinin sufficiently (Burnet and Anderson, 1947). It should be mentioned that antiserum *per se* did not affect the mobility of either virus-treated or normal cells.

As far as possible, techniques for the individual viruses are summarized in Table I. Details for mumps virus are given below. RDE treatment was carried out by incubating red cells for 2 hours at 37° C. with an enzyme dilution of titre 2000 (7·5 ml. per 0·25 ml. of packed cells). It was shown that longer incubation did not result in a greater reduction in mobility.

TABLE I.—*Technical Details of the Treatment of Red Cells with Viruses.*

Virus.	Amount of virus containing allantoic fluid.		Time of treatment	Stabilization.
	Volume per 0·25 ml. packed cells.	Titre (with 1 per cent. fowl cells).		
Mumps	7·5	220	See text	See text
NDV	5–7·5	500	4 hours	Self-stabilizing
MEL	5	1000	4 ,,	Stabilized with antiserum
PR8	7·5–10	500	4 ,,	,, ,,
BEL	7·5	500	4 ,,	,, ,,
LEE	7·5	500	4 ,,	,, ,,
SW	7·5	500	8–24 ,,	,, ,,
MIL	5–7·5	500	24 ,,	,, ,,

After treatment with virus or RDE, each preparation of cells was tested both for agglutinability with the standard series of viruses, and for electrophoretic mobility as described under "Methods." The results obtained in a number of experiments with each agent are summarized in Table II. The viruses are

arranged in the table according to the order in which they fall in the red cell "receptor gradient," i.e. the order in which the agglutinability of the cell for the different strains is destroyed by virus action (Burnet, McCrea and Stone, 1946; Stone, 1947). Although it would appear from the results given that SW should fall at the end of the gradient, it has been placed before MIL because detailed study of the action of SW itself on red cells has shown the agglutinability of cells for a standard dose (5 agglutinating doses) of SW is always lost before that for MIL.

Mumps-treated cells were first prepared using mumps-infected allantoic fluid. Even after treatment for periods of up to 24 hours at 37° C. these cells were still unstable and the electrophoretic mobility showed little change from the normal value. In one experiment samples taken after incubation for 4, 8 and 24 hours gave mobility values of 1·28, 1·28 and 1·26 respectively. However, if after incubation in mumps-allantoic fluid for 4 hours or more, the cells were removed from this medium by centrifugation, resuspended in saline and held at 37° C. for several hours, they became stable, as tested at 37° C. although not at room temperature. These cells no longer agglutinated at 37° C. with the test-dose of mumps virus but still agglutinated with the other viruses, the reaction with NDV, however, often being less than with normal cells (Table II). Similar results were obtained if the cells were treated for 4 to 8 hours at 37° C. with the same amount of virus-infected allantoic fluid which had been diluted with four volumes of saline or other diluent, such as phosphate, acetate or borate buffer. The mobility values for cells treated with mumps-infected allantoic fluid, followed by incubation in saline, or with diluted allantoic fluid are combined in Table II.

With the other strains of virus the technique of treating red cells with undiluted virus-containing allantoic fluid was found to be satisfactory. The minor variations in the agglutination reactions of the treated cells which occurred in different experiments are given briefly below. The reactions shown in the tables for each strain were those found most commonly in a number of experiments.

NDV-treated cells failed to agglutinate with the test dose of mumps, NDV or MEL showed reduced agglutinability with PR8, and in some cases with BEL, but unchanged agglutinability with LEE, SW and MIL.

MEL-treated cells failed to agglutinate with mumps, NDV and MEL; the agglutinability with PR8 was reduced to a varying extent in different experiments, while reactions with the remaining viruses were unchanged.

PR8-treated cells failed to agglutinate with mumps, NDV, MEL and PR8; the agglutinability with BEL was reduced or absent, while reactions with the remaining viruses were unchanged.

BEL-treated cells failed to agglutinate with mumps, NDV, MEL, PR8 and BEL, but still reacted with LEE, SW and MIL.

LEE-treated cells failed to agglutinate with mumps, NDV, MEL, PR8 and BEL, still agglutinated with LEE, although in some cases to a reduced extent, while agglutination with SW and MIL was unchanged.

SW-treated cells failed to agglutinate with the test dose of all viruses except MIL. Agglutination with MIL was considerably reduced or, in some experiments, negative.

MIL-treated cells failed to agglutinate with the test dose of all viruses except SW and MIL.

As treatment of red cells with LEE or MIL did not destroy the agglutinability

TABLE II.—*Electrophoretic Mobility and Agglutinability of Red Cells Treated with Viruses and RDE.*

Treatment of cells.	Average agglutination with standard dose of virus.								Electrophoretic mobility (μ/sec./volt/cm.).	
	Mumps.	NDV	MEL.	PR8.	BEL.	LEE.	SW.	MIL.	Average.	Range.
Mumps	—	+	++	++	++	++	++	++	1·18 S.E. = 0·011	1·20—1·15
NDV	—	—	—	+	++	++	++	++	0·50 S.E.=0·012	0·56—0·45
MEL	—	—	—	+	++	++	++	++	0·80 S.E.=0·007	0·84—0·76
PR8	—	—	—	—	+	++	++	++	0·74 S.E.=0·009	0·80—0·70
BEL	—	—	—	—	—	++	++	++	0·75 S.E.=0·001	0·77—0·73
LEE	—	—	—	—	—	++	++	++	0·74 S.E.=0·008	0·79—0·70
SW	—	—	—	—	—	—	—	+	0·37 S.E.=0·01	0·42—0·30
MIL	—	—	—	—	—	—	++	++	0·67 S.E.=0·016	0·70—0·62
RDE	—	—	—	—	—	—	—	—	0·17 S.E.=0·004	0·19—0·16
Nil	++	++	++	++	++	++	++	++	1·30 S.E.=0·001	1·32—1·29

++ Complete agglutination. + Partial agglutination. — No agglutination.

for the test-dose of the homologous virus, attempts were made to obtain further action with these two strains by successive treatments with several changes of virus. The cells were treated with antiserum after each exposure to virus in order to remove any inert, non-eluting particles. However, cells treated in this way with either LEE or MIL showed no greater reduction in agglutinability or electrophoretic mobility than those given a single treatment only.

It is evident from the results in Table II that each virus, in addition to producing changes in the agglutinability of red cells, caused a significant reduction in their electrophoretic mobility. The extent of this change in mobility corresponded with the position of the virus in the receptor gradient in the case of mumps, MEL, PR8, BEL, LEE and MIL. Cells treated with RDE showed a very low mobility in accordance with their complete loss of agglutinability. When cells were treated with NDV, however, their mobility was reduced to a lower value than would be expected from the change in agglutinability. We consider that SW also provides an exception to the correlation in so far as it reduces the mobility considerably further than would be expected from the position assigned to it in the gradient. However, the mobility values given for MIL (and LEE) do not refer to theoretically complete enzyme actions, since the treated cells still agglutinated to practically full titre with homologous virus. Thus the question cannot be categorically answered although the big difference between the values, MIL 0·66 and SW 0·37, would suggest that SW has a further action on mobility in addition to that resulting in loss of agglutinability.

Variations in the mobility of different samples of treated cells were usually accompanied by similar variations in agglutinability. While the average mobility values for cells treated with PR8, BEL or LEE are essentially identical, values given for cells treated with the other viruses show differences which vary in magnitude. The significance of the difference, p,* between MEL and PR8, MEL and LEE, PR8 and MIL, LEE and MIL = 0·13 ; between MEL and BEL = 0·07. The significance of the difference between any other combination of two viruses in Table II is <0.05.

Further action of NDV.

In the course of work on NDV it was observed that cells treated with infected allantoic fluid showed a further decrease in mobility if they were reincubated after suspension in saline. It should be pointed out that although cells treated with NDV-allantoic fluid had become stable, some virus, termed the " cell sensitizing agent " by Burnet and Anderson (1946), was still adsorbed to their surface. These cells were not treated with antiserum as a routine procedure, since the addition of antibody only caused strong agglutination, the " cell sensitizing agent " being firmly attached to the red cell and not removable by antiserum.

Table III gives the results obtained when the standard treatment with NDV-infected allantoic fluid (4 hours at 37° C.) was followed by further incubation (4 hours at 37° C.) in various media. It will be seen that incubation in saline (normal or calcium acetate) caused a further decrease in mobility (signifi-

* Level of significance computed by formula $\frac{m_1 - m_2}{\sqrt{\sigma_1^2 + \sigma_2^2}} = \frac{\bar{x}}{\sigma}$; probability values obtained from Dunn's table (*Physiol. Rev.*, 1929, **9**, 341.)

TABLE III.—*Electrophoretic Mobility and Agglutinability of NDV-treated Red Cells after Further Incubation in a Second Medium.*

Treatment of cells.		Agglutination with standard dose of virus.								Electrophoretic mobility (μ/sec./volt/cm.).	
First (4 hours, 37° C.)	Second (4 hours, 37° C.)	Mumps.	NDV	MEL.	PR8.	BEL.	LEE.	SW.	MIL.	Average.	Range.
NDV-allantoic fluid	*Nil*	—	—	—	+	++	++	++	++	0·50 S.E.=0·012	0·56—0·45
	Normal saline of calcium acetate saline	—	—	—	—	+	++	++	++	0·36 S.E.=0·004	0·38—0·34
	NDV-allantoic fluid	—	—	—	+	++	++	++	++	0·51	··
	Normal allantoic fluid	—	—	—	+	++	++	++	++	0·52	··

TABLE IV.—*Electrophoretic Mobility and Agglutinability of Red Cells Treated with Mumps Virus Purified by High Speed Centrifugation.*

Amount of virus suspension.		Time of treatment.	Agglutination with standard dose of virus								Electrophoretic mobility (μ/sec./volt/cm.).
Volume (per 0·25 ml. packed cells).	Titre (with 1 per cent packed cells).		Mumps.	NDV	MEL.	PR8.	BEL.	LEE.	SW.	MIL.	
3 ml.	250	24 hours	—	—	—	+	++	++	++	++	0·95
2·5 ml.	1000	32 ,,	—	—	—	—	+	++	++	++	0·68

cance, $p<0{\cdot}001$) and agglutinability, whereas suspension in fresh NDV-infected allantoic fluid or in normal allantoic fluid (13-day) prevented any change.

Treatment of cells with partially purified virus.

In order to test the effect on the red cell of virus freed from the other constituents of allantoic fluid, the various strains have been prepared in partially purified form. Samples of MEL, PR8, BEL, LEE, SW and MIL were prepared by adsorbing the virus onto red cells and eluting it into saline. When red cells were treated with these eluates, the changes in mobility and agglutinability with each strain did not differ significantly from those found with infected allantoic fluid (Table II).

In the case of mumps virus it was noticed that, in order to obtain a significant effect on the electrophoretic mobility of the red cell, it was necessary to reduce the concentration of allantoic fluid present during the reaction. This was achieved either by sedimenting the cell-virus complex in the centrifuge and resuspending in saline, or by previous dilution of the infected allantoioc fluid with saline. These results indicated that the action of mumps virus on cells was inhibited by some constituent effective when the allantoic fluid was used undiluted. Preparations of mumps virus precipitated with methanol and resuspended in saline were therefore tested. The results were similar to those obtained with diluted infected allantoic fluid, average mobility 1·17. When cells were treated with virus partially purified by high-speed centrifugation (25,000 g. for 75 minutes at room temperature, deposit resuspended in saline) still lower mobility values were obtained. The results of two experiments are given in Table IV. Due to the haemolytic action of mumps virus (Morgan, Enders and Wagley, 1948) it has not been possible to carry out experiments of sufficient duration to ensure that the true equilibrium point of the reaction had been obtained.

The lower values obtained when NDV-treated cells were further incubated in saline similarly suggested the presence of some inhibitory substance in NDV-infected allantoic fluid. Preliminary experiments, using NDV purified by high speed centrifugation (25,000 g. for 2 hours at room temperature, deposit resuspended in saline) have also resulted in low values comparable with those obtained by incubation in saline.

DISCUSSION.

Hanig's finding (1948) that the action of PR8 virus causes a decrease in the electrophoretic mobility of human red cells has been extended to include all members of the series: mumps, Newcastle disease, influenza A and B viruses, and the receptor destroying enzyme of *V. cholerae* (RDE). This has enabled the virus-red cell interaction, hitherto recognizable only by changes in agglutinability, to be placed on a quantitative basis, the result of each reaction being expressible in physical terms. The question is immediately raised concerning the nature of the isogenic change on the cell surface. Little work has been done towards ascertaining the groupings on the surface which are responsible for the electrophoretic mobility of the cell. By carrying out electrophoresis of red cells in buffers of different pH values but similar conductivities, Furchgott and Ponder (1941) found that the isoelectric point of human red cells was about 1·7. The isoelectric point of the lipid, extracted from cell stromata, was found to be

about 2·6, and that of the residual protein about 4·7, suggesting that the responsible grouping was associated with the lipid fraction. The authors concluded that the cell surface was dominated by strong acid groups, and because of the high content of cephalin in the stromata, it was suggested that the phosphoric acid group of cephalin might be largely responsible for the electrokinetic behaviour. The protein present was considered not to contribute appreciably to the mobility.

Three possible explanations of the reduction in mobility by virus action have been suggested by Hanig (1948)—loss of a charged substance from the cell, change in the spatial configuration of charged groupings of the cell or adsorption by the cell of charged elements of the solvent after treatment of the cells with virus. From the work of Gottschalk and Lind (1949) it is known that a dialysable product is released following the action of viruses or RDE on the soluble inhibitor of egg white. It may be assumed that their action on red cells will also result in the liberation of a product which, however, will not necessarily be of exactly the composition. Electrophoresis of egg-white inhibitor before and after the action of viruses and RDE has not been carried out owing to difficulty experienced in purifying the inhibitor. Whether the product liberated from red cells contains a strong acid group is not known. The magnitude of the reduction in mobility of red cells following the action of viruses, and particularly of RDE when the mobility is reduced by 87 per cent, argues against the possibility of modification of the ionization of the acid grouping. If uncovering of a strong basic group which neutralizes the acid group takes place, it would follow that the liberated product itself contains an acid grouping which previously masked the basic group.

When the effect of the various viruses and RDE on the electrophoretic mobility of the red cell is compared, it is seen that the strains fall into a series which closely parallels the gradient obtained from agglutination reactions. In the latter the viruses and RDE were arranged in series so that each member destroyed the agglutinability of the cell for the virus preceding it. The phenomenon was interpreted as representing enzymic action on a progressively larger number of substrate groupings as one proceeded along the gradient from one virus to another. It is clear, however, that reduction in the electrophoretic mobility cannot be directly equated with changes in agglutinability. Comparison of the results of NDV and MEL-treated cells in Table II show that although such cells gave similar agglutination reactions, they differed markedly in their electrophoretic mobility. That the agglutinatiliby of the cell is not a direct function of the total residual charge, however, does not necessarily mean that the attraction between virus and cell is not primarily electrostatic in nature; the electrophoretic mobility measures only the net charge on the cell surface, and does not indicate the condition present at any particular point on that surface. The mobility values of NDV and SW-treated cells will be discussed more fully in later papers, but it may be said here that the values obtained are possibly the result of two actions, the second one affecting the agglutination reactions of the cell to a smaller extent than the first.

The findings with mumps and Newcastle disease virus leave little doubt that in allantoic fluid there is an inhibitor for these strains which is not destructible by the virus. In the case of mumps virus the inhibitor interfered almost completely with the action of the virus, while with NDV it appeared to block the action at an intermediate stage. There was no indication of the presence of an inhibitor for the other viruses.

Our value for the electrophoretic mobility of PR8-treated red cells (0·79—0·70 μ/sec./volt/cm.) differs considerably from the figure given by Hanig (0·6—0·5 μ/sec./volt/cm.). It is possible, however, that the adsorptive properties of a strain towards various surfaces would be influenced by its history in the laboratory. In this way sub-strains derived from the same parent stock but maintained under different conditions might vary in their effect on the red cell receptor. Such variations would be reflected in the electrophoretic mobility and agglutinability of the virus-treated red cells.

In this connection it is interesting to note that Isaacs and Edney (1950) have recently isolated a variant from a preparation of MEL virus which, though it appears antigenically identical with the standard laboratory strain of MEL, occupies a different position in the red cell receptor gradient determined by agglutination reactions and differs in its effect on the electrophoretic mobility of the cell.

SUMMARY.

The electrophoretic mobility of human erythrocytes has been determined following treatment at 37° C. with allantoic fluid preparations of mumps, Newcastle disease, influenza A or B viruses, or with the receptor-destroying enzyme (RDE) of *V. cholerae*. In each case a reduction in mobility occurred from the normal level (1·30μ/sec./volt/cm.) to values characteristic of the treating agent.

The average mobility (μ/sec./volt/cm.) of cells treated with the various agents was as follows: Mumps virus 1·18, influenza A strains—MEL 0·80, BEL 0·75, PR8 0·74; influenza B strains—LEE 0·74, MIL 0·67; Newcastle disease virus 0·50; swine influenza virus 0·37; RDE 0·17.

The order of this series closely resembles the gradient derived from the effect of the various agents on the agglutinability of the red cell. However, Newcastle disease and swine influenza viruses yielded mobility values considerably lower than expected from their position in the agglutinability gradient.

When purified preparations of virus were used most strains gave similar results. Lower mobility values, however, were obtained with mumps and Newcastle disease viruses, indicating the presence of inhibitors for these strains in allantoic fluid.

Evidence has been presented that the loss of agglutinability for viruses is not a direct function of the total residual charge on the red cell.

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ELECTROPHORETIC STUDIES OF VIRUS-RED CELL INTERACTION : ADDITIVE EFFECT OF VIRUSES OF THE INFLUENZA GROUP AND THE RECEPTOR-DESTROYING ENZYME OF *V. cholerae*.

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In the preceding paper (Ada and Stone, 1950) the effects of the various viruses and the receptor-destroying enzyme of *V. cholerae* (RDE) on the electrophoretic mobility of human red cells were compared. The mobility was reduced in each case to a level characteristic of the treating agent. The arrangement of the viruses and RDE in the order of the mobility change produced gave a series closely similar to the " receptor gradient " (Burnet, McCrea and Stone, 1946), a hierarchy formed according to the progressive destruction of red cell agglutinability.

In order to study further the degree of correlation between the electrophoretic mobility and agglutinability of red cells following exposure to virus or RDE, cells were treated successively with two different strains of virus or with one virus followed by RDE. It was hoped, furthermore, that this type of experiment would yield information concerning the nature of the virus receptor on the red cell, particularly as the readings of electrophoretic mobility provide a quantitative estimate of the change produced by the enzymic action of the virus.

A systematic study of the additive effects of two agents on the electrophoretic mobility and agglutinability of human red cells is reported in the present paper.

MATERIALS AND METHODS.

All reagents used in these experiments have been described in detail in the preceding paper (Ada and Stone, 1950).

Viruses.—The strains used were as follows:

Mumps, Newcastle disease virus (NDV); influenza A—MEL, PR8, BEL ; influenza B—LEE, MIL ; swine influenza (SW).

Red cells.—Human group O cells from a single donor.

Technique of treating red cells with viruses and RDE.

The following standard techniques were used throughout. Virus-containing allantoic fluid was mixed with packed red cells and incubated under the conditions shown.

The amount of virus required per 0·25 ml. of packed cells is expressed as the volume of fluid of given titre (haemagglutinin titre for 1 per cent fowl cells). When fluids of different titre were used the volume was adjusted to give an equivalent amount of virus.

NDV	5–7·5 ml.	Titre 500	4 hours	37° C.
MEL	5 ml.	,, 1000	4 ,,	,,
*PR*8	7·5–10 ml.	,, 500	4 ,,	,,
BEL	7·5 ml.	,, 500	4 ,,	,,
LEE	7·5 ml.	,, 500	4 ,,	,,
SW	7·5 ml.	,, 500	24 ,,	,,
MIL	5–7·5 ml.	,, 500	24 ,,	,,

NDV_p After treatment as under NDV cells were centrifuged, resuspended in acetate buffered saline (pH 6·2) containing 0·1 per cent (w/v) $CaCl_2$ and incubated for a further 4 hours at 37° C.

Mumps. The technique used is described under " methods."

RDE 7·5 ml. Titre (Burnet and Stone, 1947) 2000, 2 hours 37° C.

Stabilization of virus-treated red cells.

After treatment with any virus except mumps or NDV the cells still agglutinated when suspended in saline and it was necessary to *stabilize* them by removing residual virus with the corresponding antiserum. The lowest concentration of antiserum which gave a negative agglutination pattern when mixed with a sample of the treated cells (1 per cent suspension) was determined by titration. Serum was added to the cells in this relative amount and, after standing for 20 minutes at room temperature, the suspension was centrifuged and the cells washed with saline. (For full details see the preceding paper.)

Test for agglutinability of treated red cells.

Of a 1 per cent suspension of the treated cells 0·25 ml. volumes were mixed with equal volumes of dilutions of the test viruses containing five agglutinating doses for normal human red cells. Agglutination was read by the pattern of the cells after settling at room temperature.

Microelectrophoresis.

This was carried out at 25 $\pm$ 1° C. in a M/15 sodium potassium phosphate buffer of pH 7·35 $\pm$ 0·05. Details are given in the preceding paper.

EXPERIMENTS.

The conditions required for the action of the individual viruses and RDE have been considered in detail in the first paper of this series. In carrying out successive treatments with two agents the technique used for each action was the same as described above (under " methods "). With all strains of virus except mumps or NDV the cells were stabilized with antiserum after each treatment. The experiments will be reported in the order in which the virus used for the first treatment occurs in the agglutinability gradient. In each case sufficient red cells were treated with the first agent to enable all second treatments to be carried out in parallel. Normal red cells were treated under precisely the same conditions with the agents used in the second treatments. A sample of cells was retained as control after the first treatment only.

Since the first treatment destroyed the agglutinability of the cells for certain other viruses—the extent of this effect depending on the relative positions in the receptor gradient—it was not possible to study the additive effects of all viruses in the series. In most cases second treatments were attempted only with viruses which, from previous experiments, would be expected to agglutinate, i.e. to be adsorbed to the once-treated cells. The degree of agglutination visible during each second treatment is recorded below. As RDE is the last member of the receptor gradient it could be used in the second treatments of all cells.

It was found previously that two different mobility levels could be obtained when NDV acted on red cells, depending on the technique used. When cells were treated with undiluted NDV-infected allantoic fluid the mobility was reduced, on the average, to 0·50. This treatment will be referred to as "NDV" If these cells were further incubated after resuspension in saline, the mobility fell to 0·35. A similar value was obtained if cells were treated with NDV partially purified by high speed centrifugation. This action will be termed "NDV_p." Both types of treatment were included in the experiments described below.

Each sample of treated cells was tested both for agglutinability with the standard series of viruses and for electrophoretic mobility. The mobility readings are recorded in Tables I to VIII. In each case the values obtained after double treatment are presented together with the value obtained for the corresponding control carried out in parallel and the range of values obtained previously with the same agent (Ada and Stone, 1950). The results of agglutinability tests are not given since the reactions of cells treated with two agents did not differ significantly from those of control cells treated with the second agent only. The latter have been described in detail in the preceding paper.

Second treatment of cells pretreated with mumps virus.

Due to the haemolytic action of mumps virus (Morgan, Enders and Wagley, 1948), it was desirable that the first incubation period should be kept to a minimum. Cells were therefore incubated (8 hours at 37° C.) with a concentrated and partially purified preparation of mumps virus obtained by methanol precipitation (1·2 ml. of titre 1400 per 0·25 ml. packed cells). These cells were then treated with the other viruses or RDE according to the standard techniques. Strong agglutination similar to that with control cells occurred during treatment with all strains except NDV. There was no visible agglutination with NDV even in the control tube under the conditions used.

It can be seen from the results in Table I that, with minor exceptions, the mobility readings for cells given the double treatment were within the range of the single treatment values.

It was found previously that mumps virus caused a greater reduction in electrophoretic mobility if preparations partially purified by high speed centrifugation were used. Attempts were therefore made to use this technique for the initial treatment of cells in these experiments. However, as the method involves long incubation periods the cells haemolysed freely when treatment with a second agent was attempted and no mobility readings could be obtained.

Second treatment of cells pretreated with NDV.

After preliminary treatment with NDV in the form of infected allantoic fluid, samples of cells were incubated with all strains of virus, except mumps, or

TABLE I.—*Electrophoretic Mobility of Red Cells Treated with Mumps Virus followed by other Viruses or RDE.*

First treatment.	*Nil.*	NDV.	NDV_p.	MEL.	PR8.	BEL.	LEE.	SW.	MIL.	RDE.
Nil . .	—	0·52 (0·56–0·45)	0·35 (0·38–0·34)	0·82 (0·84–0·76)	0·71 (0·80–0·70)	0·73 (0·77–0·73)	0·74 (0·79–0·70)	0·41 (0·42–0·30)	0·62 (0·70–0·62)	0·19 (0·19–0·16)
Mumps .	1·20 (1·20–1·15)	0·58	0·35	0·85	0·69	0·73	0·74	0·42	0·63	0·22

The single values are those obtained in the present experiment ; figures in brackets show the range obtained previously (Ada and Stone, 1950).

TABLE IIA.—*Electrophoretic Mobility of Red Cells Treated with NDV followed by other Viruses or RDE.*

First treatment.	*Nil.*	MEL.	PR8.	BEL.	LEE.	SW.	MIL.	RDE.
Nil . . .	—	0·80 (0·84–0·76)	0·73 (0·80–0·70)	0·77 (0·77–0·73)	0·75 (0·79–0·70)	0·37 (0·42–0·30)	0·68 (0·70–0·62)	0·17 (0·19–0·16)
NDV . . .	0·51 (0·56–0·45)	0·52	0·48	0·38	0·40	0·35	0·42	0·26

TABLE IIB.—*Electrophoretic Mobility of Red Cells Treated with NDV_p followed by other Viruses or RDE.*

First treatment.	*Nil.*	MEL.	PR8.	BEL.	LEE.	SW.	MIL.	RDE.
Nil . . .	—	0·82 (0·84–0·76)	0·71 (0·80–0·70)	0·75 (0·77–0·73)	0·71 (0·79–0·70)	0·34 (0·42–0·30)	0·62 (0·70–0·62)	0·17 (0·19–0·16)
NDV_p . . .	0·38 (0·38–0·34)	0·38	0·36	0·35	0·34	0·29	0·36	0·26

TABLE III.—*Electrophoretic Mobility of Red Cells Treated with MEL Virus followed by other Viruses or RDE.*

First treatment.	*Nil.*	PR8.	BEL.	LEE.	SW.	MIL.	RDE.
Nil . . .	—	0·76 (0·80–0·70)	0·77 (0·77–0·73)	0·71 (0·79–0·70)	0·39 (0·42–0·30)	0·68 (0·70–0·62)	0·16 (0·19–0·16)
MEL . . .	0·80 (0·84–0·76)	0·72	0·72	0·71	0·42	0·72	0·15

with RDE. Full agglutination occurred with PR8, BEL, LEE, SW and MIL, but no agglutination was visible with MEL. The results in Table II*a* show that in this set of experiments the values after double treatment were much lower than the control values, except in the case of SW and RDE. The figure for NDV plus SW agrees very well with the SW control while the value for NDV followed by RDE is somewhat higher than the RDE control. In the case of NDV followed by MEL or PR8 the results are similar to the NDV control, indicating that little if any further action has taken place. The other strains caused a further decrease in mobility, although this was least marked with MIL despite the low control figure.

When cells were first treated with NDV according to the technique resulting in greater reduction in mobility (NDV_p), they showed no visible agglutination on the addition of MEL or PR8. Agglutination was progressively stronger with the other strains—partial with BEL, slightly less than the control with LEE and complete with SW and MIL. The mobility readings are shown in Table II*b*. The mobility of NDV_p-treated cells was further reduced after exposure to PR8, BEL, LEE, SW or MIL virus or to RDE. The value for NDV_p plus SW was significantly lower, and that for NDV_p plus RDE higher, than the corresponding controls. In the other cases, the initial value of the NDV_p-treated cells was already much lower than the control for the second virus.

Second treatment of cells pretreated with MEL virus.

Following treatment with MEL virus, cells were incubated with PR8, BEL, LEE, SW or MIL virus or with RDE. Partial agglutination occurred with PR8, full agglutination with the other viruses. It can be seen from the readings in Table III that all agents caused a further reduction in mobility of MEL-treated cells. Except with MIL where the figure was again higher, all values were within the control range for the second agents.

Second treatment of cells pretreated with PR8 virus.

Cells treated first with PR8 were reincubated with BEL, LEE, SW, MIL, or RDE. Full agglutination occurred with all viruses. While no further reduction of mobility resulted from the action of BEL or LEE (Table IV), exposure to MIL caused a decrease in mobility from the PR8 control value to one only slightly above the MIL control. There was a marked reduction to values corresponding with the controls after the action of SW or RDE.

TABLE IV.—*Electrophoretic Mobility of Red Cells Treated with PR8 Virus followed by other Viruses or RDE.*

First treatment.	*Nil.*	BEL.	LEE.	SW.	MIL.	RDE.
Nil . . .	—	0·77 (0·77–0·73)	0·73 (0·79–0·70)	0·37 (0·42–0·30)	0·68 (0·70–0·62)	0·16 (0·19–0·16)
PR8 . . .	0·76 (0·80–0·70)	0·78	0·76	0·35	0·70	0·18

Second treatment of cells pretreated with BEL virus.

After treatment with BEL, cells were incubated with LEE, SW, MIL or RDE. Full agglutination occurred with the three viruses. In all cases there was a

further decrease in mobility of the BEL-treated cells, to values which agreed well with the corresponding controls in the case of LEE and RDE, but were somewhat above the controls when SW or MIL were used (Table V).

TABLE V.—*Electrophoretic Mobility of Red Cells Treated with BEL Virus followed by other Viruses or RDE.*

First treatment.	*Ni*	LEE.	SW.	MIL.	RDE.
Nil	—	0·73 (0·79–0·70)	0·39 (0·42–0·30)	0·68 (0·70–0·62)	0·17 (0·19–0·16)
BEL	0·77 (0·77–0·73)	0·72	0·46	0·74	0·18

Second treatment of cells pretreated with LEE virus.

When cells pretreated with LEE were incubated with SW, MIL or RDE, full agglutination occurred with the two viruses. There was no decrease of mobility following the action of MIL, and the value after SW action, though considerably reduced, was higher than the control figure. RDE brought the mobility to a figure within the control range (Table VI).

TABLE VI.—*Electrophoretic Mobility of Red Cells Treated with LEE Virus followed by other Viruses or RDE.*

First treatment.	*Nil.*	SW.	MIL.	RDE.
Nil	—	0·39 (0·42–0·30)	0·68 (0·70–0·62)	0·17 (0·19–0·16)
LEE	0·73 (0·79–0·70)	0·45	0·75	0·19

Second treatment of cells pretreated with SW virus.

Following treatment with SW, cells were incubated with MIL virus or RDE. Only a trace of agglutination occurred on the addition of MIL and there was no evidence from the mobility readings (Table VII)) that any enzymic action took place. RDE, however, caused a marked reduction in mobility to a value slightly lower than the control.

TABLE VII.—*Electrophoretic Mobility of Red Cells Treated with SW Virus followed by other Viruses or RDE.*

First treatment.	*Nil.*	MIL.	RDE.
Nil	—	0·68 (0·70–0·62)	0·18 (0·19–0·16)
SW	0·39 (0·42–0·30)	0·41	0·15

Second treatment of cells pretreated with MIL virus.

When MIL-treated cells were incubated with RDE, the mobility was sharply reduced to a value only slightly above that of the RDE control (Table VIII). As treatment with MIL virus did not destroy the agglutinability for SW virus (Ada and Stone, 1950), it was also possible to test the effect of MIL followed by SW on cell mobility. Agglutination occurring on the addition of SW to MIL-treated cells was equivalent to that observed with the control cells. The mobility was

reduced from the MIL level of 0·68 to 0·45, a value slightly higher than the figure for SW acting on normal cells (0·39).

TABLE VIII.—*Electrophoretic Mobility of Red Cells Treated with MIL Virus followed by other Viruses or RDE.*

First treatment.	*Nil.*	SW.	RDE.
Nil	—	0·39 (0·42–0·30)	0·19 (0·19–0·16)
MIL	0·68 (0·70–0·62)	0·45	0·21

DISCUSSION.

The additive effect of two agents, whether both are strains of virus or a virus followed by RDE, on the red cell surface has been studied, using two methods which reveal virus action—destruction of agglutinability and alteration of electrophoretic mobility. The number of combinations available was limited by the relative positions of the viruses in the receptor gradient; only those strains which still agglutinated cells after the first treatment could be used for the second action. A strict comparison was always made with cells exposed only to the agent used in the second part of the experiment.

With all combinations of agents tested the loss of agglutinability after double treatment was the same as after exposure to the second agent alone. In most cases comparable electrophoretic mobility readings were also recorded. Thus cells treated first with either mumps, MEL, PR8, BEL, LEE or MIL virus showed a mobility similar to that of normal cells when both were incubated with any strain of virus which reacted, or with RDE. Several minor discrepancies occurred when the second treatment was carried out with a virus close in the agglutinability gradient to the strain used for the initial treatment. For example, values higher than the controls were obtained when NDV followed mumps virus (0·58, compared with NDV control 0·52) or SW followed LEE (0·45, compared with SW control 0·39). In these cases the degree of adsorption of virus to cells would be reduced (Stone, 1947) and it is possible that the action would have proceeded further if allowed longer than the standard time. Somewhat higher values were similarly obtained when MIL virus was used as the second agent, irrespective of the first strain used. For example, MIL succeeding MEL or BEL gave values 0·72, 0·74 respectively, whereas the MIL control value was 0·68 in each case. These findings may be a further instance of the inefficient action of MIL virus apparent to some extent when normal cells are treated (Ada and Stone, 1950).

Mobility readings differing markedly from the control figures were obtained when cells pretreated with NDV were exposed to a second virus. This was the case using either undiluted NDV-infected allantoic fluid ("NDV") or virus freed from allantoic-fluid-inhibitor ("NDV_p"). The values in all cases, except that of NDV followed by SW, were considerably lower than the corresponding controls. When S.W. acted on NDV-treated cells, however, the mobility reading agreed closely with that of cells treated by SW alone. As was reported previously (Ada and Stone, 1950), NDV and SW are the only strains whose effect on cell mobility does not agree with their position in the gradient derived from agglutination reactions, the reduction in mobility being greater than anticipated in each case. This fact, together with the similarity of the mobility

values obtained on incubating cells with NDV followed by SW and those given with SW alone, indicates that these two strains have some additional action on red cells which is common to both, but possessed by none of the other viruses. Furthermore, as treatment of cells with RDE alone yields a value similar to that obtained with SW followed by RDE, and slightly lower than the value of NDV followed by RDE, this property must also be shared by RDE. No explanation for the high value obtained following RDE action on NDV-treated cells can be offered at the present time.

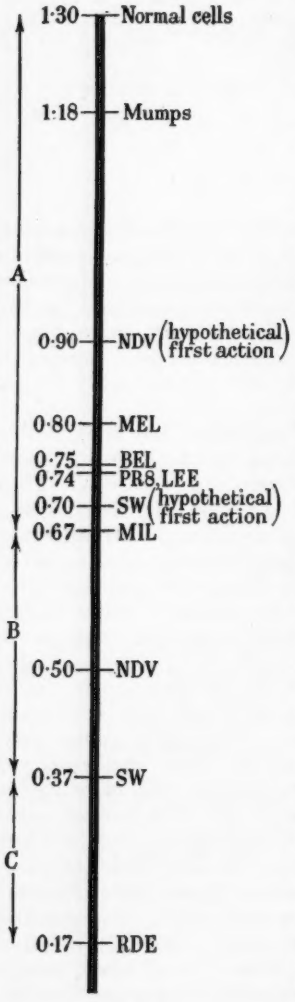

FIG. 1.

The following concept of the action of viruses and RDE on red cells is presented in an attempt to interpret the results obtained. On a graph of descending electrophoretic mobility values of red cells (Fig. 1) the levels to which the various viruses and RDE reduce the mobility have been marked at the appropriate points. It must be emphasized that the values are presented on the graph in a linear relationship purely for the sake of simplicity, and that this convention has no bearing on the hypothesis. The graph is divided into three sections, termed A, B and C. It is postulated that mumps, MEL, PR8, BEL, LEE and MIL viruses can all act upon Section A to varying extents as indicated by the mobility values of treated cells. On the other hand, NDV and SW can act upon section A to values which in the case of NDV fall between those of mumps and MEL, and in the case of SW between LEE and MIL, in accordance with their positions in the agglutinability gradient. In addition to acting upon part of Section A, both NDV and SW can act upon all of Section B. RDE acts upon the whole of Sections A, B and C.

As part of this hypothesis, values for the equilibrium points of the first actions of both NDV and SW must be postulated. While studying the haemolytic action of NDV, Burnet (1950) found that haemolysis did not occur if cells were treated with either NDV (as infected allantoic fluid) at room temperature or with virus which had been preheated at 56° C. for 30 minutes. The mobility of cells so treated for periods up to 24 hours was in the range of 0·95 to 0·85. It was inferred from these results that the haemolytic activity of the virus was concerned with a "second action" which reduced the mobility of cells from the range of 0·95 to 0·85 down to 0·50. Consequently the mobility value representing the equilibrium point of NDV action on Section A alone has been set at 0·90. In the case of SW the figure 0·70, being the mid-point between LEE and MIL, has been temporarily chosen. Investigations to obtain evidence of a second action of SW are in progress.

If cells were pretreated with NDV_p, the values obtained when any of the other viruses, including SW, were used for second treatment were significantly lower than the control values for the second virus. This could only be interpreted in the scheme outlined above as indicating that NDV under these conditions can also act upon part of Section C.

The question of the nature of the difference between the three sections arises. Two possibilities seem to exist. First, that while mumps, MEL, PR8, BEL, LEE and MIL possess one enzyme capable of acting on red cells, NDV and SW possess two (possibly three in the case of NDV) and RDE three. Or secondly, that whilst all the viruses and RDE contain only one enzyme, the actual substrate group thus being common to the three sections, the groupings in the immediate environment of the substrate vary, but can be divided into three broad classes. At the present time no clear choice can be made between the alternatives. In the first case, RDE has not yet been purified to the stage where it can be regarded as a homogeneous compound. Until such purification has been achieved, the possibility that it contains more than one enzyme remains. As regards the second possibility, it is feasible that the environment of a substrate would affect the adsorption and hence the enzymic action of particles as large as virus to a greater extent than the adsorption and activity of a smaller particle, such as RDE (Ada, French, Curtain and Laver, 1950 (in preparation)). Consequently, while RDE could act upon all three sections, the environment of the "enzymically active patch" on the virus would largely determine the number of substrates with varying environments which could be attacked.

In conclusion it must be stressed that the foregoing scheme will almost certainly have to be modified as the work progresses. It is presented here purely as the simplest working hypothesis into which the facts already established appear to fit.

SUMMARY.

Human erythrocytes have been exposed at 37° C. to the successive action of two of the following viruses; mumps, Newcastle disease (NDV), influenza A (MEL, PR8 or BEL strains), influenza B (LEE or MIL strains), swine influenza (SW), or to the action of one of these viruses followed by the receptor-destroying enzyme of *V. cholerae* (RDE). The treated cells were tested for agglutinability with the various viruses and for electrophoretic mobility and compared with control cells which had received a single treatment only with each of the agents tested.

With all combinations of agents, the loss of agglutinability of the cells after double treatment was the same as after exposure of normal cells to the second agent alone.

Cells pretreated with either mumps, MEL, PR8, BEL, LEE or MIL virus and then incubated with RDE or with any strain of virus which still caused agglutination after the first treatment, showed electrophoretic mobility values similar to those obtained when normal cells were treated with the second agent only.

Mobility readings markedly lower than those obtained following action of the corresponding second agent alone were observed when cells, treated first with NDV-infected allantoic fluid, were incubated with any virus, other than SW.

When SW acted upon NDV-treated cells, the value agreed closely with that given by SW alone. Exposure of cells pretreated with purified NDV to any virus yielded values lower than those of the controls.

When RDE acted on cells treated first with virus the mobility was reduced to the level shown by RDE control cells.

The results are discussed and a hypothesis covering the observations is suggested.

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STUDIES ON DIABETIC RATS: THE PRODUCTION OF CARDIOVASCULAR AND RENAL DISEASE IN DIABETIC RATS.

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MANY clinical reports indicate that diabetic individuals are more prone to develop vascular disease than are non-diabetics of the same age group (Rabinowitch, 1944; Root, Bland, Gordon and White, 1939; Goodof, 1945; Clawson and Bell, 1949). Warren (1930) has stated that all patients seen by him at autopsy who had had diabetes for five years or more were found to have arteriosclerosis, regardless of their age group. This statement was subsequently slightly modified (Warren, 1938), but it may still be taken as an indication of the high incidence of arteriosclerosis in diabetics. These authors also assert that the major problem in the treatment of diabetics is the predilection of these patients to develop premature and progressive vascular lesions, which lead to such complications as gangrene, blindness, cardiac insufficiency, cerebral disorders and intercapillary glomerulosclerosis. Although some clinicians believe that these dangerous complications of the diabetic state can be largely prevented by adequate control with insulin, others feel that even when diabetics are thus rigidly controlled, such vascular lesions still develop prematurely. This apparent relationship between diabetes and vascular disease has not been adequately investigated by means of controlled clinical or experimental studies. The immense difficulties attendant upon controlled clinical research on this problem can be readily visualized, and it was decided to attempt an experimental approach to it.

Perhaps the most obvious reason which has been advanced for the apparent relationship between the diabetic state and vascular degeneration is the fact that most diabetics have plasma lipid levels which are either at or above the upper limits of normality. It has been assumed and analyses have been reported to substantiate the assumption that the more adequate the control of the diabetes, the more nearly normal is the level of the plasma lipids (Joslin, Root, White, Marble and Bailey, 1946).

This possible etiological factor takes on added significance in the light of the vast experimental work on animals, in which increased plasma lipid levels have been shown to be a definite predisposing cause of atherosclerosis (Antischkow, 1933).

However, although it is accepted that increased plasma lipids do play a role in the production of experimental atherosclerosis, it is also recognized that they do not constitute the sole responsible factor. In this connection it may be of significance to note that Dragstedt (1945) has reported an increased incidence of arteriosclerosis in dogs made diabetic by complete pancreatectomy and maintained with insulin. It should be emphasized that the plasma lipids in such animals are usually in the subnormal range (Chaikoff and Kaplan, 1934).

It would appear quite possible, therefore, that the increased incidence of arteriosclerosis in diabetics is not due primarily to increased plasma lipid levels, and that some unknown concomitant of the diabetic state affecting the deposition of lipids in the vessel wall is the responsible etiological factor. Since the fundamental defect involved in this process is concerned with lipid metabolism, the influence exerted by the pancreas thereon should be examined closely. The hyperlipaemia which occurs in uncontrolled diabetes is well known. On the other hand, there is a tendency for dogs made diabetic by complete pancreatectomy to develop a hypolipaemia, in spite of which an increased incidence of vascular disease ensues (Dragstedt, 1945). In these cases plasma lipids were increased readily by the administration of choline, methionine, or by the administration of lipocaic, which is an extract made from pancreas (Dragstedt, Prohaska and Harms, 1936). Chaikoff, Entenman and Montgomery (1947) have demonstrated that the active principle in lipocaic is in all probability a proteolytic enzyme, which presumably liberates methionine and/or choline from the intestinal chyme. Apart from their dramatic effect on the level of plasma lipids, choline and methionine also prevent the abnormal accumulation of lipids in the liver. It is quite possible that these substances might also exert a similar lipotropic effect on the vascular tree. This suggestion has been investigated by several workers, and their results are discussed in the following paper (Beveridge and Johnson, 1950).

Apart from the deterrent of the tremendous difficulties involved in controlled clinical research, the decision to attempt an experimental approach was based on the premise that the tendency to vascular degeneration is not unique to human diabetics and that it may occur also in other mammalian diabetics. The choice of an experimental animal was based on the following points : Firstly, economy ; secondly, knowledge of dietary requirements ; and thirdly, ease of induction of diabetes. The white rat appeared to be the most appropriate animal, although it was realized, at that time at least, that no one had been able to induce intimal vascular disease in this animal. Just recently, however, Stubbs, Shrader and Broun (1948) and Marx, Marx, Meserve, Shimoda and Deuel (1949) have described the production of aortic lesions in the rat by dietary means.

EXPERIMENTAL.

One hundred and fourteen rats of the Wistar strain, weighing 170 to 240 g., were fasted for 48 hours. They were then made diabetic by the subcutaneous injection of freshly prepared aqeuous 5 per cent (W/V) alloxan monohydrate (Eastman Kodak) in a dosage of 185 mg. per kg. of body weight. They were given isotonic saline for drinking purposes for 48 hours. Urine samples were collected 24 hours after the injection of alloxan, and tested for reducing substances by means of Benedict's qualitative reagent. All samples gave positive results ranging from ++ to ++++.

Four groups of 24 animals each were selected and placed on special diets. The basal diet, Table I, was fed to a group of 24 normal control rats (Group I) and to a similar group of diabetic rats (Group II). A third group of animals received a diet high in fat, in which this component was changed from 10 per cent to 16 per cent mazola oil plus 53 per cent beef dripping, which, along with the cod liver oil, made a total of 70 per cent fat. Corn starch was accordingly reduced

TABLE I.—*Basal Diet.*

Crude casein	12 per cent.
Skim milk powder	12 ,,
Mazola oil	10 ,,
Corn starch	60 ,,
Cod liver oil	1 ,,
Wheat germ	1 ,,
Yeast	2 ,,
Bone meal	1 ,,
Celluflour	1 ,,

from 60 per cent to 1 per cent. The fourth group was given the same diet except that 0·5 per cent cholesterol was added at the expense of a similar amount of corn starch. Finally, the fifth group was given the basal diet, to which was added 0·5 per cent cholesterol in place of an equal amount of corn starch. The cholesterol used was a sample obtained from the Armour Laboratories, Chicago, Illinois, U.S.A. The cholesterol was converted into its amorphous form according to the procedure described by Domosi and Egyed (1939), and quoted by G. Popjak (1946). These workers using rabbits have shown that this form of cholesterol is readily absorbed from the alimentary tract. Sperry's colorimetric procedure for cholesterol (1943) indicated a purity of 81 per cent in this sample.

Those diabetic animals on the experimental diets which died during the first three weeks were replaced from the pool of diabetic animals. From time to time urine samples were tested for reducing substances to ascertain whether or not the diabetic state was still present. Where difficulty was experienced in obtaining urine specimens, samples of blood were taken from the tail and blood glucose estimations performed (Nelson, 1944). In only one instance did these tests reveal that the diabetes had not been permanent.

During the second 24 hours after the administration of alloxan, the rats were given one unit of crystalline insulin and one unit of protamine zinc insulin. Thereafter the animals were maintained on the latter preparation, the amount of which was varied from time to time depending on the rat's condition as judged by the presence or absence of ketosis and, in some cases, as indicated by blood and urine sugar determinations. It must be noted, however, that no attempt was made to control rigidly the diabetes, and the insulin dosage was made as uniform as possible for all animals and changed only when necessary. The rats of the control group were given minimal injections of protamine zinc insulin to simulate as far as possible the experimental conditions to which the test rats were subjected.

After periods of time varying from 7 to 11 months a number of animals from each group were sacrificed following a nine-hour fast. Analyses for total and free cholesterol, phospholipid and total fatty acids were carried out on both plasma and liver. The details and results of these chemical determinations are reported in the paper immediately following (Beveridge and Johnson, 1950). Blocks of tissue were taken from the heart, aorta, kidney, pancreas, liver, retina and adrenal for histological examination. A similar routine was followed for any animals that were killed in *extremis*. In the case of animals which died,

blocks of tissue were taken provided there was no gross evidence of post-mortem autolysis. In all instances, however, sections of the heart and aorta were taken. After the first 11 months the remaining rats were permitted to live out their life span so as to allow as long a time as possible for the development of vascular and other tissue lesions. The mortality curves are shown in Fig. 1. The normal control animals had by far the lowest mortality. Of the diabetic groups, that receiving the basal diet (Group II) had the longest average survival time (268 days). The diets containing high levels of fat (Group III) or cholesterol (Group V) had an adverse effect, lowering the average life span to 217 and 203 days respectively. The most detrimental diet was the ration in which both fat and cholesterol levels were elevated (Group IV). This regime permitted an average survival time of only 151 days.

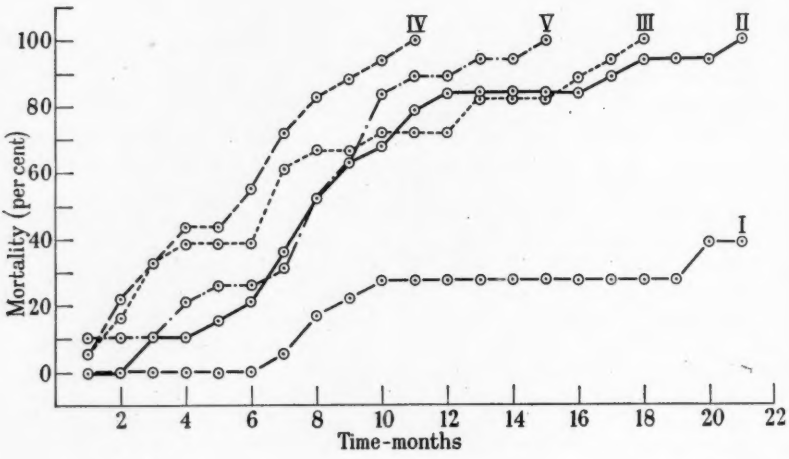


FIG. 1.

HISTOLOGICAL EXAMINATION OF TISSUES.

All tissues were fixed in 10 per cent formalin solution, and the sections were stained with haematoxylin and eosin. Weigert elastic tissue stain was applied to those sections in which vascular lesions were suspected on the basis of an examination of the haematoxylin and eosin preparation. In order to detect readily any atheromatous plaques, the aortas were split longitudinally and stained with Scharlach R according to the method of Klotz (1915). The results of the histological examination of the tissues will be taken up in the following order: Myocardium, vessels of heart, kidney and pancreas, aorta, kidney, pancreas, liver, retina, adrenal.

Myocardium.

The heart was cut in three blocks from which a total of 12 serial sections were taken. These were cut at intervals of 400 microns. In all diabetic groups there was present a degree of myocarditis, which varied from simply a collection of polymorphonuclear leucocytes and chronic inflammatory cells (Fig. 2) to areas of scarring ranging in size from one to several millimetres (Fig. 3, 4). The incidence of myocarditis in the different groups was as follows: Group I, 0 per cent; Group II, 29 per cent; Group III, 56 per cent; Group IV, 40 per cent; Group V, 21 per cent.

Vessels of heart, kidney and pancreas.

A thorough examination of the many sections taken revealed the presence of only a few vascular lesions in which the intima was involved. The abnormalities observed ranged from what appeared to be simply a cellular proliferation (Fig. 5, 6, 7, 8) to a diffuse hyaline thickening of the intima (Fig. 9); that in Fig. 10 is seen as a greatly swollen and vacuolated structure simulating in appearance the picture of atherosclerosis in human subjects. The incidence of vessel lesions was as follows: Group I, 29 per cent; Group II, 24 per cent; Group III, 13 per cent; Group IV, 27 per cent; Group V, 16 per cent. These figures were calculated on the basis of the finding of one or more lesions in the many sections prepared from each animal.

A type of arteriosclerosis previously described by Wilens and Sproul (1938*a*) in which there were markedly hypertrophied and vacuolated muscle cells immediately beneath the intima was found in both diabetic and control rats. However, since the intima was not involved, no importance has been attached to this finding.

Aorta.

The aorta was cut into three blocks, which were imbedded together on edge. Twelve sections were taken at intervals of 400 microns. The treatment of the aortas with Scharlach R failed to reveal the presence of any atheromatous plaques. On microscopic examination of the sections no intimal lesions were observed.

Kidney.

The renal lesions observed were by far the most striking of the tissue changes which occurred. Grossly the renal capsule usually stripped quite readily, but the surface was often markedly pitted with a few small cysts.

A pyelonephritis of varying severity was present in almost 100 per cent of the diabetic animals. In the milder cases the microscopic sections showed simply collections of chronic inflammatory cells in the kidney parenchyma. In the more severe examples these collections of inflammatory cells were larger and more numerous. There was an increased amount of fibrous tissue, particularly between the tubules. The tubules showed varying degrees of degeneration, the lumina in some cases being obliterated by collections of pale, finely reticulated cells. In others the tubules were markedly dilated and contained pseudo-colloid

material (Fig. 11). In the more severely damaged kidneys there was periglomerular, fibrosis accompanied by a marked thickening of Bowman's capsule and adhesions between the visceral and parietal layers. Frequently tufts of the glomeruli were hyalinized and the glomeruli were not infrequently entirely hyalinized (Fig. 12).

The incidence of pyelonephritis was as follows : Group I, 63 per cent ; Group II, 100 per cent ; Group III, 93 per cent ; Group IV, 77 per cent ; Group V, 92 per cent. The figure for Group IV is lower than those recorded for the other diabetic animals and is almost undoubtedly due to the markedly shorter life span of this group.

Pancreas.

With one exception all the diabetic rats showed a marked decrease in the number and size of the pancreatic islets.

Liver.

The microscopic sections of the liver showed nothing remarkable except a marked accentuation of the foamy appearance of the cells in Groups IV and V, presumably due to an increase in the deposition of cholesteryl ester, to which further reference will be made in the following paper.

Retina and adrenal.

The sections of retina and adrenals appeared to be normal.

EXPLANATION OF PLATES.

FIG. 2.—Section of myocardium from a diabetic rat fed a diet high in fat for 4 months. Foci of inflammatory cells and small areas of fibrosis are present. × 50.

FIG. 3.—Section of myocardium from a diabetic rat fed a diet high in cholesterol for 10 months. A fairly large scarred area is shown. × 50.

FIG. 4.—Section of myocardium from a diabetic rat fed a diet high in both fat and cholesterol for 6 months. There is fibrosis with calcification, and almost the entire wall of the ventricle is involved. × 50.

FIG. 5.—Section of kidney vessel from a diabetic rat fed a diet high in both fat and cholesterol for 7 months. The lesion involving the intima is comprised of a fibrillar material, and although three or four nuclei may be discerned no definite cell outlines can be distinguished. × 160.

FIG. 6.—Section of a kidney vessel from a diabetic rat fed the basal diet for 7 months. The lesion is similar to that described in Fig. 5 except that there is some vacuolation. × 160.

FIG. 7.—Section of a coronary vessel from a diabetic rat fed a diet high in fat for 4 months. The greatly thickened walls contain cells possessing nuclei which are variably but not peripherally situated and cytoplasm of a granular and reticulated appearance. × 250.

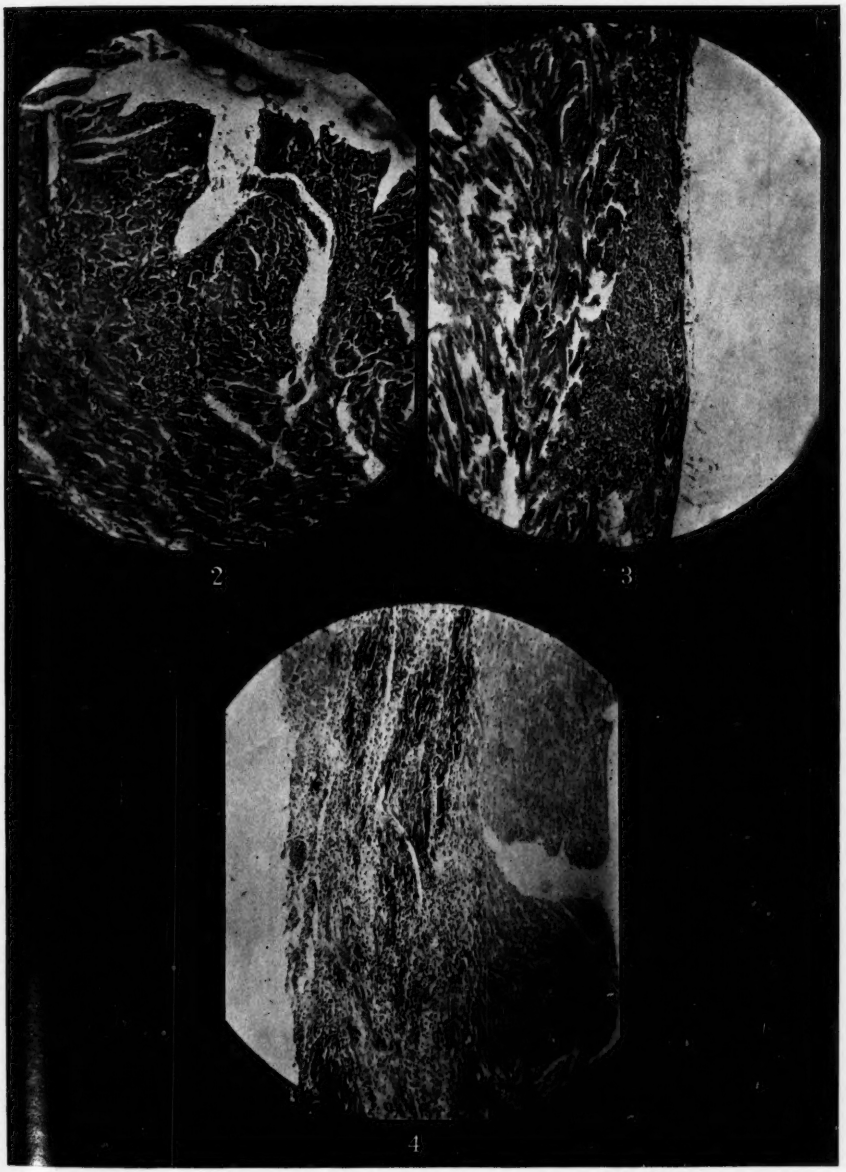
FIG. 8.—Section of s coronary vessel from a diabetic rat fed a diet high in cholesterol for 10 months. The internal elastic lamina is fragmented. The lesion is composed of fibrillar material in which only one or two cells can be seen, and these are vacuolated with peripherally placed nuclei, × 160.

FIG. 9.—Section of a coronary vessel from a control rat fed the basal diet for 16 months. The internal elastic lamina is fragmented and the intima is diffusely thickened, presenting a very finely granular appearance, and no nuclei other than those of the endothelial cells can be detected. × 160.

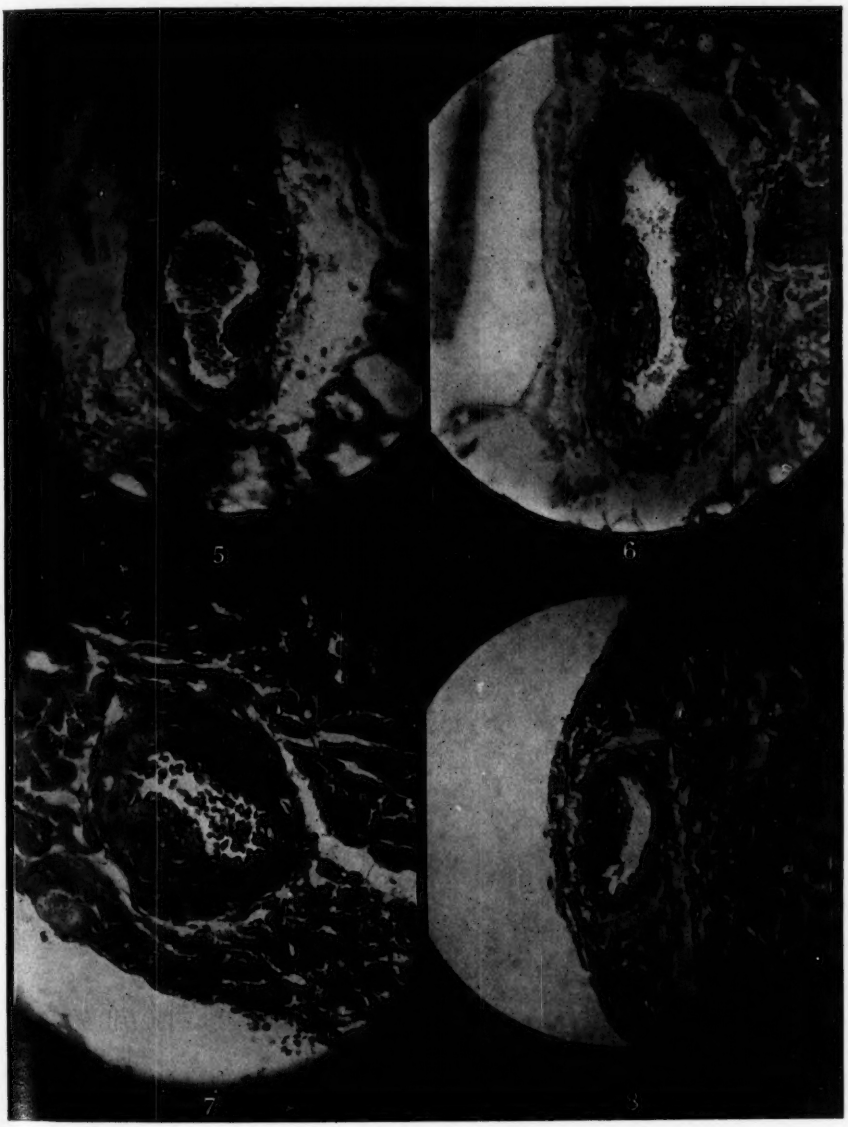
FIG. 10.—Section of a coronary vessel from a control rat fed the basal diet for 21 months. The internal elastic lamina is fragmented, and the intima is greatly thickened, with much vacuolization. The only nuclei seen in the intima are those of the endothelial cells. × 160.

FIG. 11.—Section of kidney from a diabetic rat fed the basal diet for 16 months. It is presented to show the " pseudo-colloid " material in the greatly dilated tubules. × 50.

FIG. 12.—Section from same kidney as in Fig. 11. It shows some of the typical findings in chronic pyelonephritis—partially and completely hyalinized glomeruli, widely dilated tubules, some of which contain pseudo-colloid casts, periglomerular thickening, fibrosis and foci of inflammatory cells, mostly of the round cell type. × 50.



Beveridge and Johnson



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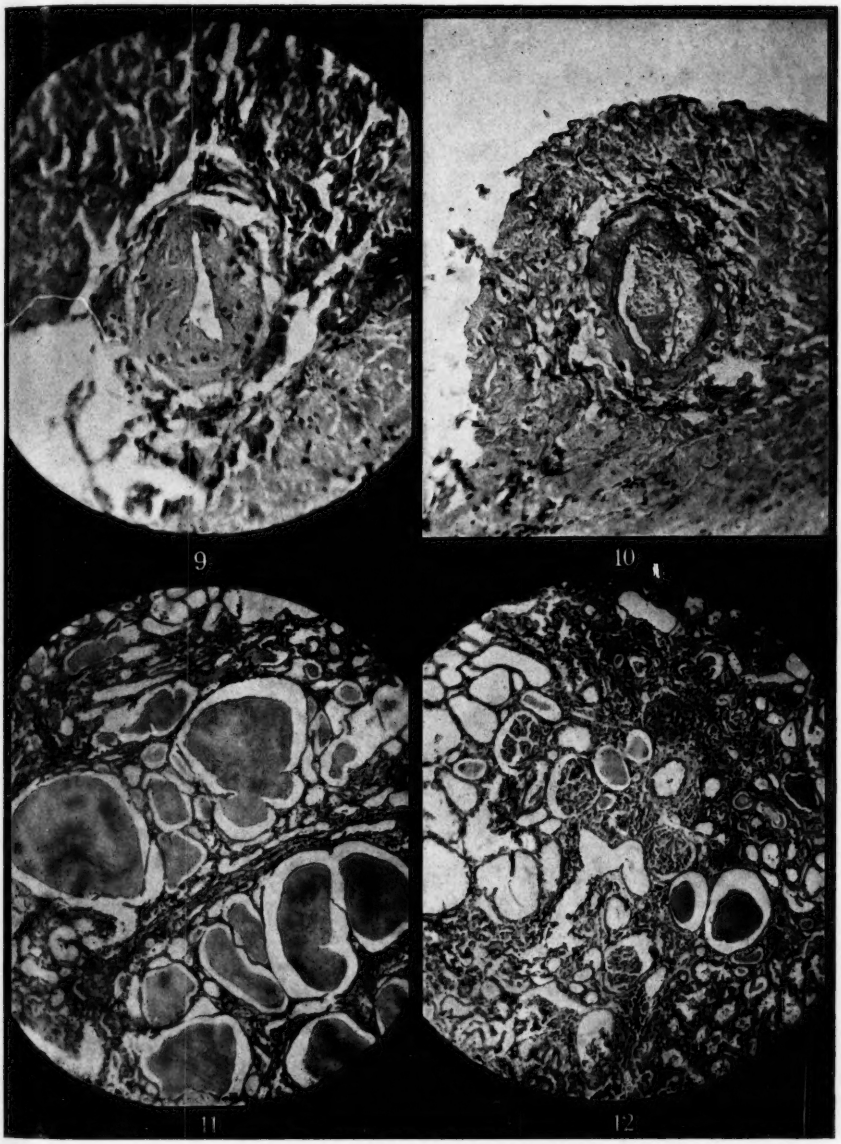

Beveridge and Johnson.

DISCUSSION.

When the plan of this experiment was originally drawn up, it was intended that a certain number of animals from each group be sacrificed at specific intervals. However, the paucity of vascular lesions found in animals so sacrificed early in the experiment led to the abandonment of this plan at the end of approximately one year. In the hope that the incidence and degree of severity of the vascular lesions might increase the longer the animals were kept under experimental conditions, all the remaining diabetic animals were permitted to live out their life span. These circumstances make the evaluation of the results among the groups of diabetic rats most difficult.

It may be recalled that no myocarditis was found in the animals of the control group, whereas an incidence of up to 56 per cent was found in the diabetic rats. On the other hand, Wilens and Sproul (1938*a*), who have published the most extensive study on pathology in the rat, found an incidence of myocarditis in normal rats ranging from 11·1 per cent. at an age under 400 days up to an incidence of 65·6 per cent at an average age at death of 702 days. In conclusion, despite the report by Wilens and Sproul, it would appear that, in this work, the incidence of myocarditis was definitely increased in the diabetic rats.

It is notable that in their study on 487 normal rats, 221 of which were males, Wilens and Sproul failed to find any coronary lesions in which the intima was involved. This finding may be contrasted with that obtained here in which 8 such lesions were observed in 92 rats. Twelve animals exhibited intimal lesions either in the kidney or the pancreas. Although the total incidence of these vascular abnormalities was approximately the same in all groups, including the control animals, it is of interest to note that of those sacrificed in the early part of the experiment, the control group did not exhibit any of these lesions. This may indicate simply that the diabetic state caused an earlier appearance of vascular degeneration such as has been shown to occur in humans. The unexpected finding of intimal abnormalities in the control rats, in view of the well-known resistance of this species to the development of such vascular lesions, warrants further investigation.

Wilens and Sproul (1938*b*) have described also renal lesions consisting of healed pyelonephritis, arteriosclerotic scars and scattered hyalinized glomeruli. The total incidence of these was 27 per cent, but they added that they were uncommon in rats under 700 days, an average age far exceeding that of the animals in this study.

The kidney damage observed in the diabetic animals of this experiment was of much greater severity and incidence than that reported above. In addition, the renal lesions observed in the control group were minimal in nature and occurred to a lesser extent. These findings lead to the conclusion that the diabetic state had a profound effect in augmenting the production of kidney lesions. Although the hyalinization of the glomerular tufts was probably caused by the chronic pyelonephritic process, nevertheless the possible etiological role of the diabetic state in the production of this lesion cannot be summarily dismissed. The critical appraisal of the results obtained in this work are complicated by the initial toxic effect of the diabetogenic dose of alloxan on the tissues. In order to determine the degree of this tissue damage, 15 rats were made diabetic with alloxan in the usual way. One animal died within 48 hours and the rest were sacrificed as follows: 5 at 2 days, 4 at 7 days, and 5 at 14 days.

An examination of the myocardial sections from animals sacrificed at 2 and 7 days revealed albuminous degeneration in some cases. At 14 days no lesions were noted. The kidney sections, on the other hand, revealed a varying degree of albuminous degeneration of the tubules at 2, 7 and 14 days. Although it seems scarcely conceivable that the degree of albuminous degeneration observed in the animals sacrificed shortly after the administration of alloxan could lead to the cardiovascular and renal lesions observed some months later, nevertheless it is a possibility which cannot be entirely disregarded.

One of our associates, Dr. J. C. Paterson, suggested that the severe pyelonephritis seen in the diabetic animals might have been secondary to a marked haemoglobinuria such as was described by Gyorgy and Rose (1948) in alloxanized rats previously fed diets low in tocopherols. This possibility was investigated, but no haemoglobinuria was found and the erythrocyte counts remained within the normal range.

SUMMARY.

Cardiovascular and renal lesions have been produced in rats made diabetic with alloxan and maintained for periods ranging from 7 to 22 months. These lesions comprised (*a*) myocarditis, (*b*) marked chronic pyelonephritis, and (*c*) lesions involving the intima of coronary, renal and pancreatic arteries.

Similar vascular and kidney lesions were noted in control animals.

The authors are greatly indebted to Professor J. C. Paterson for his critical appraisal of many of the microscopic sections, and to Professor E. M. Watson for his constructive criticism and advice.

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STUDIES ON DIABETIC RATS: THE EFFECT OF DIABETES AND OF DIET UPON THE PLASMA AND LIVER LIPIDS OF RATS.

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THE data herein presented were obtained during an experiment designed to ascertain whether or not cardiovascular and renal lesions could be induced in rats made diabetic with alloxan (Beveridge and Johnson, 1950). In view of the well-known role of plasma lipids in the production of experimental atherosclerosis (Anitschkow, 1933; Page, 1945), it was realized that a knowledge of plasma lipid levels was essential in properly assessing the cause of any vascular lesions which might be produced. Further, in view of the studies of Chaikoff and Kaplan (1934) and Kaplan and Chaikoff (1935) on the effect of pancreatectomy in dogs in decreasing plasma lipid levels and increasing liver lipid levels, it was thought that a determination of the lipid patterns of these tissues of the alloxanized rats might similarly reveal some interesting relationships.

It may be reiterated here that the factors which prevent the abnormal accumulation of lipids in the liver might also prevent similar accumulations in the vascular tree. This theory has been investigated by several workers, with results which were not always in agreement. Steiner (1938*a*, 1938*b*) reported that choline given along with cholesterol appeared to delay but not to prevent the appearance of atherosclerosis in rabbits, and that the administration of choline alone produced a resolution of aortic lesions previously induced by the feeding of cholesterol. Similar findings were published by Herrmann in a series of papers (1946*a*, 1946*b*, 1946*c*, 1947), in which he described the administration of choline or methionine to hens with a resultant drop in cholesterol levels in the aortas. On the other hand, Baumann and Rusch (1938) failed to observe any effect of choline on the prevention of atheroma in rabbits fed a diet high in cholesterol. However, these workers fed daily amounts of choline which varied from 1/6 to 1/3 as much as that fed in supplementary form by Steiner. Furthermore, Steiner's work demonstrated clearly the delayed development of atheromatous plaques in his animals by killing and examining them at intervals from 40 to 100 days. With one exception those animals fed cholesterol alone and killed within 80 days developed aortic atheromata, whereas those given cholesterol plus choline failed to develop such lesions. After 100 days choline no longer exerted its protective action. It is not surprising, therefore, that Baumann and Rusch, supplementing with much lower levels of choline, failed to demonstrate any beneficial effect of this substance in the prevention of atherosclerosis in rabbits at the end of a four-month experimental period.

It might also be noted that Leary's (1941) hypothesis for the pathogenesis of atherosclerosis involves a preliminary infiltration of the Kupffer cells with

cholesteryl ester. This process implies an overall increase in liver cholesteryl ester and, on the basis of work reported by Best, Channon and Ridout (1934) and of data presented here, presumably the neutral fat fraction would be increased also. It may be of significance with respect to the foregoing that choline, methionine and inositol are active in preventing this abnormal accumulation of lipids in the liver, and hence, if Leary's hypothesis is valid, a constant adequate supply of these lipotropic substances should aid in preventing the development of atheroma in the vascular system.

Such considerations indicated the advisability of determining the lipid patterns of both plasma and liver in animals in which the development of vascular lesions was to be investigated.

EXPERIMENTAL.

The experimental conditions have been described in a previous paper (Beveridge and Johnson, 1950).

At intervals between 7 and 11 months animals from each group were sacrificed after a 9- to 10-hour fast. Analyses for total and free cholesterol, phospholipid and total fatty acids were carried out on plasma and liver. In the latter case the weighed tissue was thoroughly homogenized with ten volumes of acetone in a Waring Blendor for 30 seconds. The mixture was transferred quantitatively to a 250 ml. centrifuge pot with the aid of hot absolute alcohol. This homogenate was centrifuged and the supernatant poured into a volumetric flask of 500 ml. capacity. The residue was re-extracted by suspension in five volumes of 95 per cent alcohol. The sample was then heated for 10 minutes in a water bath at 80° C. $\pm$ 2°, and excessive loss of solvent was prevented by means of a glass air condenser. The mixture was centrifuged and the supernatant transferred to the volumetric flask. This extraction was performed four times. The above process had been previously shown by Lucas (personal communication, 1945) to effect an extraction of 99·5 per cent or more of the lipids present, and this point also had been confirmed by us. The combined supernatants were made up to volume. Aliquots of appropriate size were then taken for lipid analysis.

Although some slight modifications were made in the methods for determining the lipids, the procedures followed were essentially those described by Boyd (1938) for Bloor's (1928) technique for total fatty acids, by Sperry and Brand (1943) for total cholesterol, by Sperry (1943) for the Schoenheimer-Sperry method for free cholesterol, and by Sinclair and Dolan (1942) for phospholipid. In the latter case, however, the phospholipid phosphorus was determined according to the procedure of Beveridge and Johnson (1949).

All these methods yielded closely agreeing replicate values on samples of lipid extract. In Sperry's procedure for free cholesterol, the individual stirring rods used throughout each determination to mix precipitating solutions and to suspend digitonide precipitates with wash liquids were found to be most cumbersome, and were discarded in favour of the following procedure. It was found that the precipitating solutions could be adequately mixed by swirling the tubes, and that the suspension of the digitonide precipitates in the wash liquids could be readily effected by the moderately vigorous discharge of these liquids against the precipitates from a syringe. The washing of the digitonide precipitates was facilitated by the choice of aliquots which did not yield bulky precipitates.

The details of the experimental diets fed to the various groups of rats are

given in the previous paper (Beveridge and Johnson, 1950). In brief outline, however, Group I was comprised of 24 normal rats which were fed an adequate basal diet. The animals of Group II to V inclusive were made diabetic by treatment with alloxan. Group II was fed the basal diet; Group III received a diet containing 70 per cent fat; Group IV was given a diet similar to Group III, but containing in addition 0·5 per cent cholesterol; and Group V received the basal diet plus 0·5 per cent cholesterol.

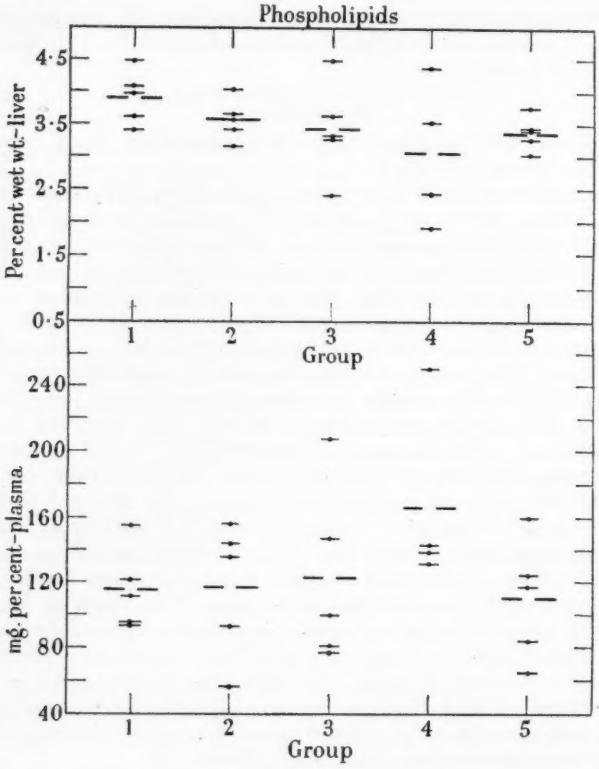

FIG. 1.

In every group there are values from 5 rats presented, with the exception of Group IV, in which values from 4 animals are given. These results are expressed in graphic form and, where dots appear to run together, this represents closely agreeing values for two or more animals. The cross bars represent averages.

Phospholipids.

The phospholipid levels in plasma and liver are illustrated in Fig. 1. Neither the diabetic state (Groups I and II) nor the variation in diet (Groups II and III,

II and IV, P = 0·18) could be shown to have wrought any significant change in plasma phospholipid as determined by the usual statistical tests.

The diabetic state caused a slight decrease in liver phospholipid concentration (Groups I and II, P = 0·17), and the addition of fat (Groups II and III), fat plus cholesterol (Groups II and IV) and cholesterol supplements (Groups II and V) have accentuated this decrease due, presumably, to increases in cholesterol and neutral fat without concomitant increases in phospholipid. A comparison of Groups I and V yielded a P value of 0·04. The explanation advanced for the decrease in liver phospholipid concentration is supported by an examination of

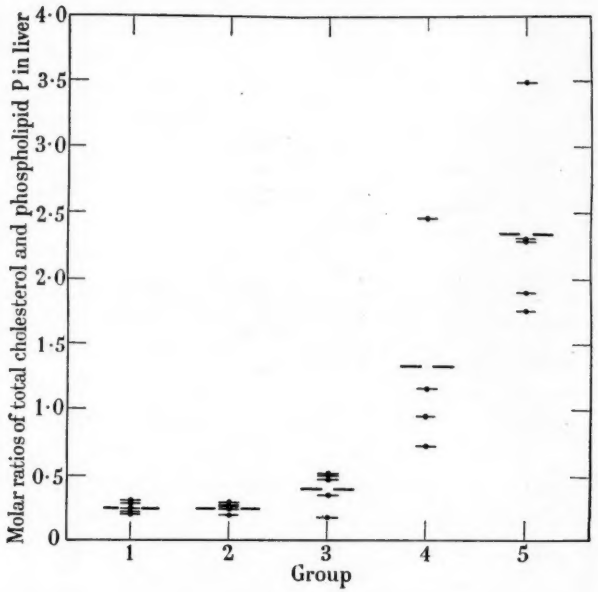

FIG. 2.

the molar ratio of total cholesterol and phospholipid phosphorus (Fig. 2). A consideration of the plasma and liver phospholipids indicates the presence of a trend toward an inverse relationship of phospholipid concentration in these tissues. A statistical examination of the data revealed, however, that this trend could not be accepted as significant since $r = -0{\cdot}351$, corresponding to a P of 0·08, which is just outside the accepted limit of significance, 0·05.

Total Fatty Acids.

The values for total fatty acid levels are depicted in Fig. 3. The production of diabetes has led to a slight but not significant increase in plasma total fatty acids as indicated by a comparison of Groups I and II (P = 0·21). The addition

of the fat supplement alone (Groups II and III) and with cholesterol (Groups II and IV) yielded extremely variable results in which the average values have been increased. These increases could not be shown to be significant. The addition of the cholesterol supplement alone has had no effect (Groups II and V).

With respect to the liver total fatty acids, the diabetic state produced no change (Groups I and II). The addition of the fat supplement both with and

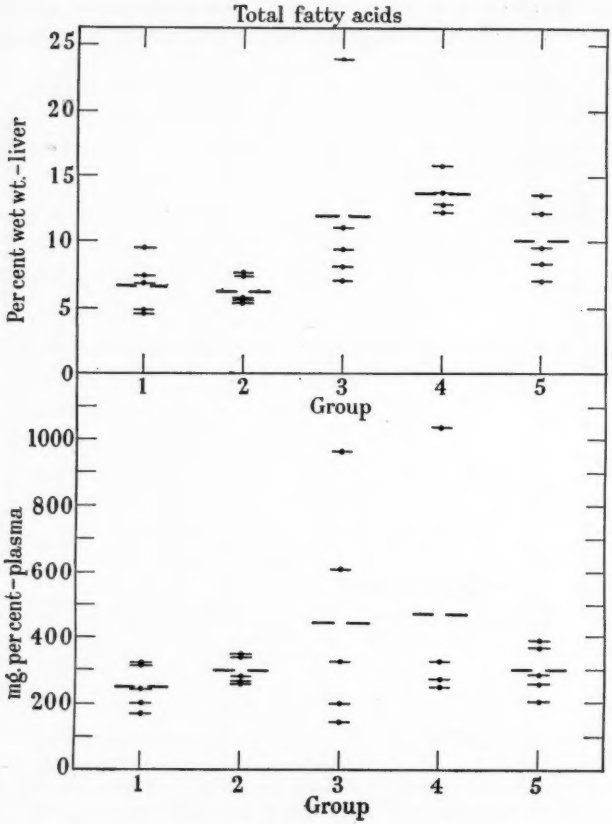

FIG. 3.

without cholesterol (Groups II and IV and Groups II and III respectively) has led to significant increases. The significance of the difference between Groups II and IV is obvious from the distribution seen on the graph ($P = <0{\cdot}01$). In the comparison of Groups II and III, one aberrantly high value in the latter was omitted, a change which actually decreased the difference between the two means. An analysis of the residual data yielded a P value of 0·03, as compared with a value of 0·12 when the aberrantly high figure was included. This anomalous

result is due to the fact that the high value in Group III made the pooled variance extremely high, thus leading to a low t value, and hence to a correspondingly higher value for P. The addition of cholesterol alone also elevated the total fatty acids (Groups II and V, $P = 0{\cdot}02$).

It may be noted that high average plasma fatty acids were accompanied by

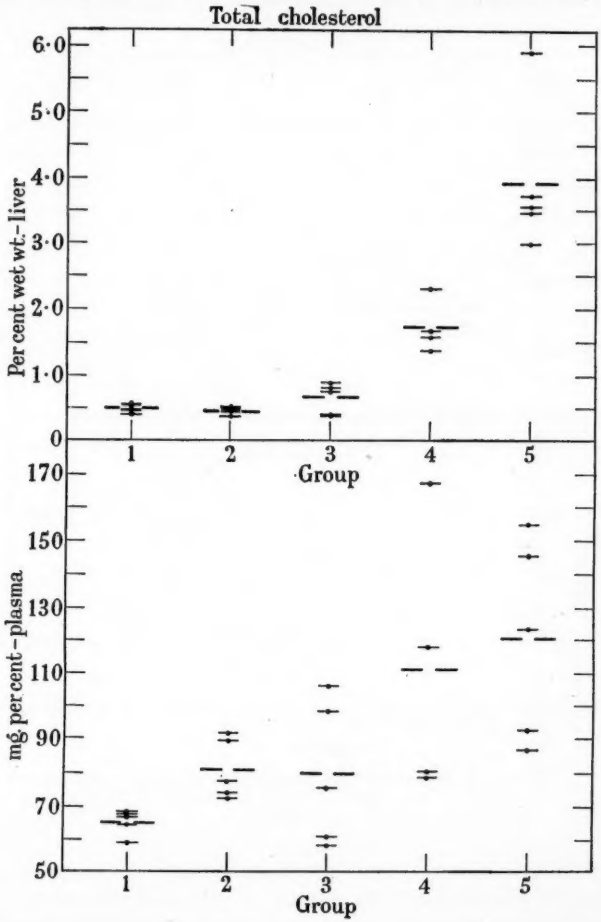

FIG. 4.

similar high levels in the liver. A correlation of $r = +0{\cdot}679$, corresponding to $P = <0{\cdot}01$, indicated that the positive correlation between plasma and liver fatty acids levels was highly significant.

Total cholesterol.

The levels of total cholesterol in plasma and liver are illustrated in Fig. 4.

A comparison of the values from Groups I and II indicated that the induction of diabetes has effected a highly significant increase in the total plasma cholesterol ($P = <0{\cdot}01$). The addition of the fat supplement has wrought no change (Groups II and III), whereas the addition of cholesterol alone (Groups II and V, $P = 0{\cdot}02$) or along with the fat supplement (Groups II and IV) has brought about a marked increase in plasma cholesterol.

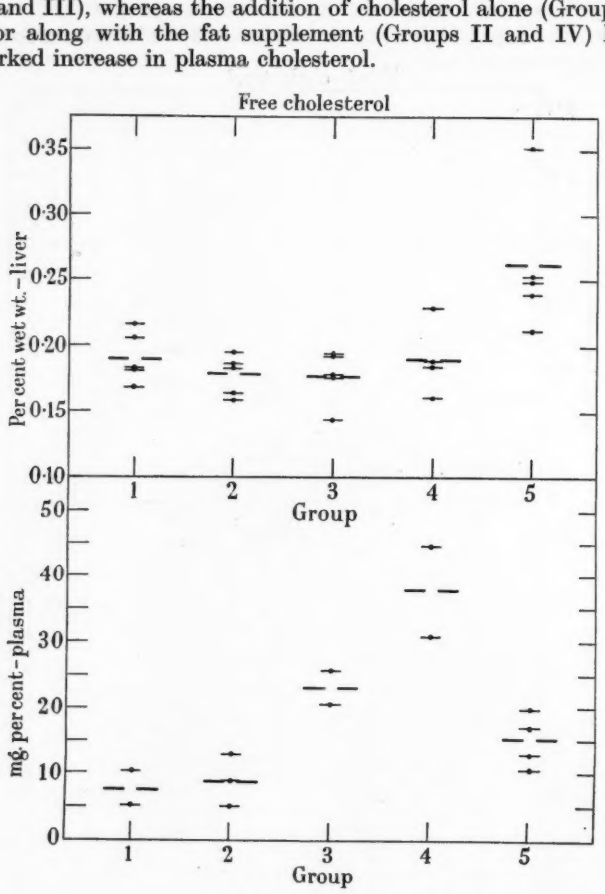

FIG. 5.

In the liver no change has been effected by the diabetic state (Groups I and II). Similarly, the addition of fat has not produced an increase which can be shown to be significant (Groups II and III, $P = 0{\cdot}07$). On the other hand, the cholesterol supplement alone (Groups II and V, $P = <0{\cdot}01$) and with fat (Groups II and IV) has brought about significantly higher cholesterol levels. It would appear from a consideration of the data that the addition of the fat supplement was not responsible for any large elevation of the liver cholesterol. Indeed, it may be noted that the addition of cholesterol to the basal diet has induced a much greater increase in liver cholesterol than the combined supplements of

cholesterol and fat (Groups IV and V, $P = <0{\cdot}01$). This phenomenon may possibly be explained by a decreased absorption of cholesterol from the high fat diet.

The trend for high plasma cholesterol levels to accompany high liver cholesterol levels appears obvious from the graph (Fig. 4), and is further supported by the high coefficient of correlation $= + 0{\cdot}518$ corresponding to a $P < 0{\cdot}01$.

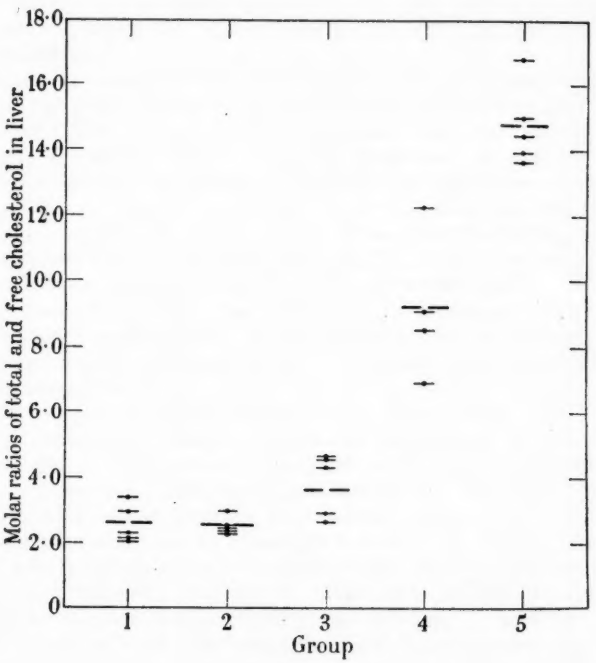

FIG. 6.

Free cholesterol.

The values obtained for free cholesterol are shown in Fig. 5. The results for free cholesterol levels in plasma do not merit discussion because of the paucity of estimations. This defection was due to an unexplained failure of colour development in a series of our determinations.

The only treatment which caused a marked change in liver-free cholesterol was the addition of cholesterol to the basal diet (Groups II and V, $P = <0{\cdot}01$).

An examination of the molar ratios for total and free cholesterol in the liver (see Fig. 6) indicates that the pronounced increase in total cholesterol observed in the livers of Groups IV and V was due primarily to a markedly increased proportion of cholesteryl ester.

Neutral fat.

The values for liver neutral fat shown in Fig. 7 are extremely variable, but indicate that the addition of the fat supplement has effected significant increases in the groups on the diets high in fat (Groups III and IV). A comparison of the results from Groups II and IV gave a P value of $<0{\cdot}01$. When the widely aberrant value of 22 per cent is discarded from Group III, a device which actually

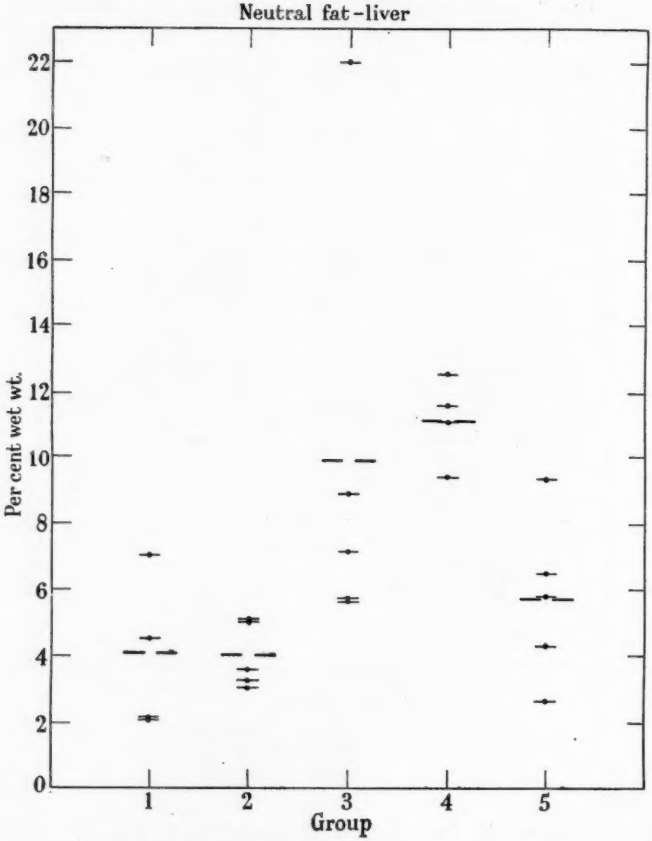

FIG. 7.

brought the means of Groups II and III closer together, an analysis of the residual data yielded a P value of 0·01.

From time to time animals were killed in *extremis* and the liver and plasma subjected to the usual lipid analyses. It was soon apparent, however, that the lipid patterns of such animals were markedly divergent from those described above. Representative of these data are the following figures for three such animals from Group IV (the average figures for the sacrificed animals are given in

brackets): Plasma phospholipid—295, 264 and 341 mg. per cent (166 mg. per cent); plasma total fatty acids—662, ... and 410 mg. per cent (474 mg. per cent); plasma total cholesterol—203, ... and 251 mg. per cent (111·4 mg. per cent); plasma free cholesterol—44·8, 64·9 and 45·5 mg. per cent (37·9 mg. per cent).

DISCUSSION.

Due to the relatively small number of intimal lesions observed in the rats that were sacrificed and on which lipid analyses were performed, no conclusions could be drawn regarding the influence of plasma lipid patterns on the development of those lesions.

An investigation similar to that reported here, but using the rabbit, an animal susceptible to the development of atherosclerosis, was carried out by Duff and McMillan (1949). The degree of atherosclerosis was less in the alloxanized animals despite a greater degree of visible lipaemia. A statistic analysis of the ratio of free to ester cholesterol, obtained by calculation from their data, was performed by us, and it was found that the difference between the two groups was highly significant, $P = <0{\cdot}01$. Even after discarding the extremely high ratios obtained from the diabetic animals, No. 1 and 2, as outlined in Tuttle and Satterley's book on the *Theory of Measurements* (1925), the probability remained less than 0·01. Thus the proportion of cholesterol in the ester form was therefore significantly greater in the control group, in which there was also found the greater degree of atherosclerosis. Whether or not these two phenomena are related remains to be shown, but it may be significant in this regard to draw attention to the report of Gibbs, Buckner and Bloor (1933), who found that in diabetes with pronounced atherosclerosis the proportion of cholesterol in the esterified form was 10 to 15 per cent above the values in non-diabetics.

The effect of feeding diets high in cholesterol on the liver lipid pattern is similar to that seen in experiments with normal control rats fed diets high in cholesterol and somewhat low in lipotropic factors. In contrast to the work of Okey (1933), who found that the free cholesterol in the liver did not change when rats were given 1 per cent cholesterol in the diet, the results obtained by us showed a marked increase in this fraction (Fig. 5, Groups II and V). It should be pointed out, of course, that this worker utilized weanling rats and fed the diet for a period of three months, whereas adult rats which were diabetic and fed for periods up to 11 months were utilized in the experiment reported here. Furthermore, the details of the diet used by Okey were not given, hence it is impossible to make any critical comparison of the results from the two investigations.

Chanutin and Ludewig (1933) fed rats diets high in carbyhodrate or in fat and containing 2·5 per cent cholesterol for periods of time varying from 10 to 30 days. They concluded from their analytical results that diets high in carbohydrate may accelerate and diets high in fat may inhibit the deposition of cholesterol in the liver of the rat. Despite the differences in diet and in experimental conditions, the data reported here support the conclusions of Chanutin and Ludewig that diets high in fat tend to inhibit the deposition of free and ester cholesterol fractions in the livers of rats fed diets rich in this sterol (Fig. 4 and 5, Groups IV and V).

The administration of diets high in cholesterol to diabetic rats described in

this work has resulted in a large increase in total plasma cholesteryl, most of which is comprised of cholesteryl ester. This effect is similar to that described by Cook and McCullough (1939) and Horlick and Havel (1948), who showed that plasma total cholesterol values were greatly increased in normal rats fed diets high in cholesterol. It would appear, therefore, with respect to the foregoing comparisons that the response of rats to diets high in cholesterol has been unchanged following the induction of diabetes by alloxan.

In conclusion the behaviour of the liver and plasma lipids in these alloxanized rats appeared to be somewhat the same as that in partially depancreatized diabetic dogs and quite dissimilar to that in totally depancreatized dogs in which the plasma lipids decrease to low or subnormal levels and liver lipids increase markedly (Chaikoff and Kaplan, 1934; Kaplan and Chaikoff, 1935).

SUMMARY.

1. With the exception of an increase in total cholesterol, the diabetic state had no effect on the level of plasma lipids in rats maintained with insulin and in the post-absorptive state.

The addition of the fat supplement caused only one change in plasma lipids, namely that the total fatty acids were increased. This increase could not be shown to be significant.

It is doubtful if any synergistic effect was exerted by combined supplementation with fat and cholesterol, the increase in plasma fatty acids and total cholesterol in Group IV being undoubtedly attributable to the supplementation with fat and cholesterol respectively.

The addition of the cholesterol supplement alone resulted in a marked elevation of plasma total cholesterol.

2. The presence of the diabetic state did not effect any change in the liver lipid pattern.

The addition of the fat supplement resulted in a marked elevation of liver neutral fat and a slight increase in cholesterol ester, which is largely masked by the increase in liver size due to the infiltration with neutral fat.

The increase in neutral fat and cholesteryl ester in Group IV may be attributed to the supplementation with fat and cholesterol respectively.

The addition of cholesterol alone raised the liver cholesterol to an extremely high level, more than an eight-fold increase over that found from feeding the basal diet. This alteration was largely accounted for by an elevation of the esterified form. There was significantly less cholesterol deposited in the liver on a diet high in fat and in cholesterol than on a diet containing a normal amount of fat and a high level of cholesterol.

3. High plasma fatty acid levels accompany high liver fatty acid levels. A similar relationship was observed for the plasma and liver total cholesterol fraction.

We are indebted to Messrs. P. Gaskell and J. McLean for technical help in the performance of some of the analyses reported in this paper.

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EVIDENCE FOR THE PRODUCTION OF CIRCULATING ANTIBODIES BY HOMOGRAFTS OF LYMPHOID TISSUE AND SKIN.

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It has been shown by Medawar (1944, 1945, 1946) that, in the rabbit, the breakdown of a homograft of skin occurs much more rapidly if the recipient animal has received either a prior homograft of skin, or prior intradermal injections of a suspension of leucocytes, from the same donor. It has therefore been argued that skin homografts are effectively antigenic, and that there exists an antigen common to leucocytes and skin. Similar evidence has also been adduced of the antigenic nature of thyroid homografts in the guinea-pig. (Woodruff and Woodruff, 1950.)

The present investigation was designed to throw further light on the question of antibody production by homografts and, in particular, to determine whether there is evidence for the existence of antibodies in the serum of animals which have received homografts.

The procedure adopted was suggested by the work of Chew and Lawrence (1937) and Cruickshank (1941), who obtained an antilymphocytic serum by injections of heterologous ground lymph-node tissue.

METHODS.

Thirty Wistars aged between 6 and 7 months and 30 black and white hooded rats aged between 7 and 8 months were used. The males weighed between 230 and 300 g., and the females between 180 and 260 g.

Ten Wistars received homografts of lymph-node, and a further 10 received homografts of skin, from a black and white donor of the same sex, a separate donor being used for each recipient. The remaining animals were used as controls. The selection was a random one except that each group of 10 consisted of 5 males and 5 females.

Each of the grafted animals was bled and killed after the grafts had been *in situ* for 21 days, and 2·5 c.c. of the serum thus obtained was injected intraperitoneally into the corresponding black and white donor. Leucocyte counts were made in the latter before, and at intervals after, the injection.

Each Wistar in the control group was bled similarly, and 2·5 c.c. of the serum was injected into one of the black and white controls of the same sex. Again white blood counts were made before and after injection.

It was found essential to standardize carefully the various procedures as follows :

Lymph node grafts.

Under ether anaesthesia 2 lymph nodes, having a total weight of approximately 0·1 g., were removed from the neck of the donor. A minute piece of each was kept for section to confirm that it was in fact lymph node, and the remainder was then quartered with a pair of cataract knives. The 8 pieces thus obtained were then grafted subcutaneously to the recipient, 2 pieces being placed together in each of 4 sites beneath the abdominal skin.

Skin grafts.

The technique of pinch-grafting described by Medawar (1944) was followed. Under ether anaesthesia 2 pieces of skin, each approximately 1 cm. in diameter, were removed from each recipient, and replaced by pieces of the same size from the donor. The grafts did not quite correspond to the " fitted " grafts described by Medawar (1945), because owing to the elasticity of the skin the raw area became appreciably larger than the graft.

Preparation of serum.

To minimize the risk of bacterial contamination, blood was obtained with a sterile syringe and needle direct from the heart, after the thorax had been opened under general anaesthesia. The blood was allowed to remain in a sterile centrifuge tube at room temperature overnight, and the serum was separated aseptically next morning. In three of the controls the blood clot and residual serum were cultured and found to be sterile.

Leucocyte counts.

The animal was lightly anaesthetized with ether and blood obtained by incising transversely one of the veins of the tail with a sharp knife. The sample was not taken until bleeding was free and the first drop of blood was never used. The first incision was made near the tip of the tail, the second in the opposite vein and about 1/4 in. more proximally, the third in the original vein but more proximally still, and so on. To avoid errors due to rhythmic fluctuations in the leucocyte count, the first count was always made at 9.30 a.m. The second count was made at 10 a.m., and was followed immediately by the injection of serum. Thereafter counts were made at 10.30, 11.00 and 11.30 a.m., 12 noon, 12.30, 1.00 and 2.00 p.m.

Total counts.—The blood was diluted in the usual manner in a leucocyte pipette, the same pipette being used for successive counts in the same animal. After preliminary shaking by hand the mixture was expelled into a glass tube of 1/4 in. bore which was closed at one end. The open end was then sealed in a flame, and the tube attached to a mechanical mixer of the rotating drum type. Mixing was continued for 10 minutes at 28 r.p.m. (the drum being 6 in. in diameter and the tube fixed at an angle of 45° to its long axis), as preliminary investigations had shown that under these conditions the distribution of the cells in the subsequent haemocytometer count corresponded with remarkable accuracy to the theoretical Poisson distribution (Table I).

In the experiment proper, 2 sample drops of the diluted blood were counted

separately in a Neubauer haemocytometer, an area of 4 sq. mm. being counted for each drop. The total number of cells counted ranged from 124 to 1450, corresponding to counts of 3410 to 40,010 cells per c.mm., so that the standard error should be between 11·1 and 38·2 cells, i.e., between 9·0 per cent and 2·6 per cent (Student, 1907). In calculating the cell count allowance had to be made for the fact that the dilution was some 10 per cent greater than would have been the case if the mixing had been done entirely in the pipette.

TABLE I.—*Comparison of Observed and Expected Cell Frequencies in Trial Haemocytometer Count.*

Number of cells per small sq. (1/400 sq. mm.)	Frequency observed.	Frequency expected (Poisson distribution).	χ^2.
0	1	1·3	
1	4	6·7	1·13
2	21	17·8	0·57
3	34	31·6	0·18
4	42	41·9	0·00
5	36	44·5	1·62
6	41	39·4	0·06
7	31	29·9	0·04
8	23	19·8	0·52
9	16	11·7	1·58
10	5	6·2	
11	2	3·0	
12	0	1·3	1·61
13	0	0·5	
14	0	0·2	
15	0	0·1	
			7·31

Mean number of cells per small square = 5·309 = m.
Total frequency = 256. $256.e^{-m} = 1·266$.
$\chi^2 = 7·31$. Degrees of freedom = 9. $P > 0·5$.

Differential counts.—These were made on stained blood films by a modification of the battlement count of MacGregor, Richards and Loh (1940). Owing to the fact that the distribution of leucocytes is uneven and that the pattern is not the same for polymorphonuclear and mononuclear cells, it is extremely difficult to arrive at any reliable estimate of the standard error. Alternative methods of counting, namely, counting all the leucocytes in a small stained film and making a combined total and differential count in the haemocytometer, were therefore investigated, but found to be impracticable in view of the large number of counts to be done. It was, therefore, decided to persist with the battlement count on stained films, but to standardize the details of the procedure so that different counts could be validly compared.

Films were made on ordinary microscope slides (3 in. × 1 in.) by placing a drop of blood 0·5 cm. from one end of the slide, and drawing it into a film with a glass spreader 2 cm. wide and held at an angle of 30° to the plane of the slide. Great care was taken to use as nearly as possible the same amount of blood for each film, and this amount was such that the film extended right to the distal end of the slide. Films which did not fulfil this requirement, which appeared uneven or which did not have clean and reasonably straight edges, were rejected and fresh films made. Satisfactory films were stained with Leishman's stain.

and fine lines were then ruled with a sharp needle as shown in Fig. 1. Counting was done under the oil-immersion objective (1/12 in.) following a battlement pattern of the dimensions shown in Fig. 2. The long segments of the battlement extended from the edge of the film to the ruled line. The length of the short segments corresponded to a rotation of the knob of the mechanical

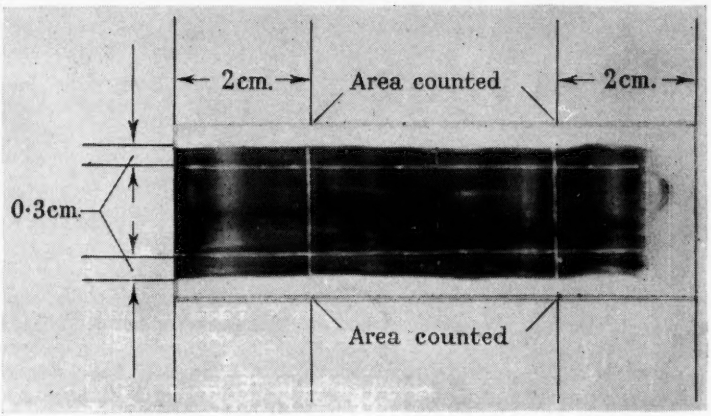

FIG. 1.—Photograph of typical blood film showing method of ruling and area counted. (Lines exaggerated for purposes of illustration.)

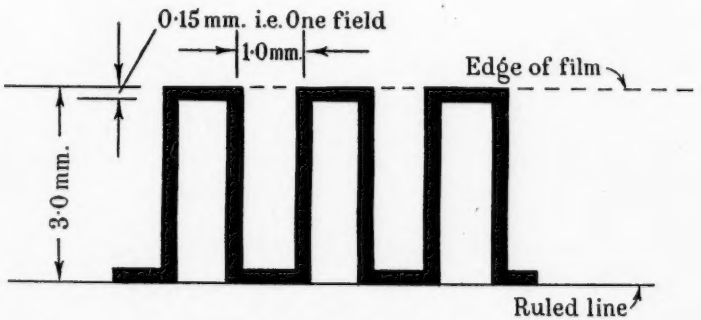

FIG. 2.—Details of battlement count. × 10.

stage through 90°, and to enable this angle to be judged accurately a matchstick was fixed radially to the knob with plasticine. Counts were made on both edges of the film, proceeding from left to right on the top edge and from right to left on the lower edge. The cells were classified simply as (1) nucleated red cells, (2) polymorphonuclear leucocytes, (3) non-polymorphonuclear leucocytes. In the last category the vast majority were lymphocytes, since rat's blood

TABLE II.—*Leucocyte Counts in Black and White Rats Before and After Intraperitoneal Injection of 2·5 c.c. Serum from Normal Wistars.*

Number of animal.	Sex.	Pre-injection counts.			Post-injection counts.							L.R. min. Ratio of lowest lymphocyte count after injection to mean pre-injection lymphocyte count.	L.R. 4. Ratio of lymphocyte count 4 hours after injection to mean pre-injection lymphocyte count.	P.R. max. Ratio of highest polymorph count after injection to mean pre-injection polymorph count.	P.R. 4. Ratio of polymorph count 4 hours after injection to mean pre-injection polymorph count.
		1 (− ½ hr.).	2 (− 0 hr.).	Mean.	1 (+ ½ hr.).	2 (+ 1 hr.).	3 (+ 1½ hr.).	4 (+ 2 hr.).	5 (+ 2½ hr.).	6 (+ 3 hr.).	7 (+ 4 hr.).				
210	M.	L. 10,860	9,960	10,410	7,290	9,020	10,690	9,790	8,860	8,030	7,100	0·682	0·682	1·20	1·20
		P. 9,170	7,850	8,510	5,960	8,140	9,110	9,410	9,680	9,960	10,220				
223	M.	L. 7,360	6,370	6,865	5,340	6,240	6,050	4,590	5,060	5,610	6,530	0·668	0·952	2·03	1·81
		P. 2,310	3,590	2,950	4,020	3,990	4,950	6,080	5,830	5,940	5,350				
224	M.	L. 6,070	6,230	6,150	6,230	5,780	5,830	6,070	6,160	6,650	7,100	0·939	1·154	1·59	1·26
		P. 4,210	3,340	3,775	5,980	5,340	4,620	4,210	4,460	4,400	4,730				
252	M.	L. 5,730	4,510	5,120	3,960	4,700	8,660	4,510	5,450	5,500	5,390	0·773	1·053	4·18	3·85
		P. 4,350	4,290	4,320	5,480	7,180	14,740	18,040	17,710	17,880	16,610				
257	M.	L. 4,940	5,340	5,140	5,040	5,180	4,170	4,570	5,180	4,320	4,690	0·811	0·912	1·47	1·47
		P. 4,630	3,880	4,255	3,520	3,380	4,990	5,280	5,080	6,220	6,020				
258	F.	L. 8,930	6,550	7,740	5,920	6,180	4,950	6,670	5,370	5,900	4,690	0·606	0·606	2·68	2·04
		P. 9,310	5,810	7,560	8,160	8,890	10,610	16,320	20,250	19,180	15,460				
259	F.	L. 6,280	5,170	5,725	5,870	3,980	3,970	4,510	4,610	4,680	3,740	0·653	0·653	2·11	0·96
		P. 5,980	4,730	5,355	6,260	6,140	6,480	11,280	9,280	9,000	5,120				
260	F.	L. 4,800	3,800	4,300	5,170	4,680	4,070	2,840	4,390	4,260	2,900	0·660	0·675	4·56	3·48
		P. 2,850	2,200	2,525	3,250	6,850	11,390	8,530	9,780	11,510	8,700				
264	F.	L. 4,540	4,850	4,695	3,700	4,780	4,180	2,900	2,920	2,940	3,040	0·617	0·647	3·23	2·57
		P. 2,910	3,825	3,370	2,400	5,070	8,800	10,900	9,110	8,210	8,660				
265	F.	L. 4,780	5,390	5,085	7,380	4,650	3,510	3,260	2,390	2,300	4,000	0·453	0·787	4·50	4·10
		P. 3,170	2,510	2,840	1,850	7,030	7,770	8,440	9,410	12,780	11,600				
Mean		L. 6,429	5,817	6,123	5,590	5,519	5,608	4,971	5,039	5,020	4,918	0·686	0·812	2·76	2·26
		P. 4,889	4,203	4,546	4,688	6,201	8,346	9,849	10,059	10,508	9,247				
		T. 11,318	10,020	10,669	10,278	11,720	13,954	14,820	15,098	15,528	14,165				

Note.—L., P., T. denote the "lymphocyte," polymorph and total leucocyte counts respectively, in cells per c.mm.

contains few, if any, true monocytes, and we shall for convenience refer to them in future simply as " lymphocytes."

Four hundred cells were counted initially, but, if these did not include 200 or more lymphocytes, counting was continued till this number was reached.

From the total and differential counts the absolute numbers of polymorphonuclear cells and " lymphocytes " per c.mm. were calculated (working to 3 significant figures), and these values are recorded in the tables. The total white-cell count was obtained by simply adding the absolute polymorph and " lymphocyte " counts. It differed slightly in some cases from the actual haemocytometer count, since the latter included any nucleated red cells present in the sample.

RESULTS.

The results are set out in Tables II, III and IV. The absolute polymorph and " lymphocyte " counts are shown separately, and the last three columns show respectively the ratio of the lowest " lymphocyte " count after injection to the mean pre-injection " lymphocyte " count (designated L.R. min.), the ratio of the " lymphocyte " count 4 hours after injection to the mean pre-injection " lymphocyte " count (designated L.R. 4), the ratio of the highest polymorph count after injection to the mean pre-injection polymorph count (designated P.R. max.), and the ratio of the polymorph count 4 hours after injection to the mean pre-injection polymorph count (designated P.R. 4).

It will be seen that in each group of animals the " lymphocyte " count fell after injection, while the polymorph count, after a temporary fall in some cases, rose considerably. The fall in the " lymphocyte " count was, however, very distinctly greater in the animals which received serum from the recipients of grafts (Groups II and III) than in the controls (Group I), and usually persisted longer. In the case of animal No. 238 (Table IV) the count 4 hours after injection was unfortunately lost owing to the tube containing the sample for the total count breaking while on the mechanical mixer.

In calculating the mean value of L.R. min. it has, however, seemed reasonable to include this animal, since whatever the count at 4 hours, the value of L.R. min. for this animal could not exceed the figure shown (0·297), and had the final count actually reduced this figure, this would have merely strengthened the conclusion we have drawn.

In calculating P.R. max. on the other hand similar reasoning is not applicable, and this animal has therefore been excluded from the calculation.

Student's " t " test (Fisher, 1936) has been used to compare statistically the values obtained from the three groups of animals. The results of the calculations are shown in Table V, from which the following conclusions appear :

1. There is no significant difference in the mean pre-injection counts in the three groups, i.e. there is no reason to doubt that the controls and the two groups of experimental animals belong to the same " population."

2. The values of both L.R. min. and L.R.4 are very significantly lower in the animals which received serum from recipients of either lymph-node or skin grafts than in the controls.

3. The absolute value of the " lymphocyte " count 4 hours after injection is significantly lower (taking $P. < 0{\cdot}05$ as the level of significance) in the animals injected with serum from recipients of skin grafts than in the controls, despite

TABLE III.—*Leucocyte Counts in Black and White Rats Before and After Intraperitoneal Injection of 2·5 c.c. Serum from Wistars "Immunized" by Homografts of Lymph Node.*

Number of animal.	Sex.	Pre-injection counts.			Post-injection counts.							L.R. min. Ratio of lowest lymphocyte count after injection to mean pre-injection lymphocyte count.	L.R. 4. Ratio of lymphocyte count 4 hours after injection to mean pre-injection lymphocyte count.	P.R. max. Ratio of highest polymorph count after injection to mean pre-injection polymorph count.	P.R. 4. Ratio of polymorph count 4 hours after injection to mean pre-injection polymorph count.
		1 (− ½ hr.).	2 (− 0 hr.).	Mean.	1 (+ ½ hr.).	2 (+ 1 hr.).	3 (+ 1½ hr.).	4 (+ 2 hr.).	5 (+ 2½ hr.).	6 (+ 3 hr.).	7 (+ 4 hr.).				
181	M.	L. 7,350	7,050	7,200	5,660	4,500	3,500	3,650	3,300	3,300	3,700	0·458	0·514	3·67	3·61
		P. 3,500	3,700	3,600	8,140	8,450	9,000	8,500	11,450	13,200	13,000				
215	M.	L. 7,670	7,060	7,365	4,540	4,070	3,310	3,170	3,450	3,470	3,260	0·432	0·443	1·39	1·20
		P. 2,960	3,700	3,330	4,630	3,900	4,140	3,440	3,850	4,280	4,000				
216	M.	L. 6,900	6,110	6,505	2,810	4,180	4,030	3,670	3,320	2,700	2,420	0·372	0·372	3·72	1·11
		P. 3,150	3,290	3,220	11,990	9,770	8,820	9,800	9,130	6,350	3,560				
300	M.	L. 9,380	7,510	8,445	6,750	5,860	2,750	4,680	5,680	3,740	4,760	0·327	0·564	1·64	1·50
		P. 11,930	9,650	10,790	12,750	12,060	7,070	12,040	17,730	11,470	16,250				
302	M.	L. 10,230	9,150	9,690	9,430	6,220	3,600	3,320	4,020	3,990	5,910	0·343	0·609	2·18	2·07
		P. 6,280	4,820	5,550	3,680	5,740	8,180	8,010	11,545	12,100	11,470				
284	F.	L. 6,710	7,060	6,885	5,970	6,970	7,410	13,390	8,680	7,070	4,480	0·650	0·650	4·10	3·42
		P. 3,580	2,760	3,170	2,390	3,920	9,150	13,010	10,930	10,250	9,040				
286	F.	L. 4,430	3,120	3,775	2,150	3,270	5,390	3,260	4,420	2,520	1,760	0·466	0·466	5·09	2·55
		P. 3,710	3,560	3,635	2,120	4,540	11,990	9,500	18,440	11,400	9,240				
288	F.	L. 5,790	4,850	5,320	4,410	4,640	2,920	2,720	2,940	3,900	2,920	0·509	0·549	1·77	1·77
		P. 7,060	4,700	5,880	4,370	5,130	7,660	8,670	8,810	10,015	10,400				
290	F.	L. 10,140	8,510	9,325	11,150	6,130	3,890	5,780	9,860	5,760	3,520	0·377	0·377	2·56	2·28
		P. 3,560	3,360	3,460	2,830	6,570	5,840	8,150	8,840	8,590	7,880				
316	F.	L. 15,870	15,620	15,745	13,750	12,500	11,970	12,110	10,995	11,700	4,500	0·285	0·285	2·09	1·50
		P. 7,130	7,605	7,368	9,650	9,525	9,505	10,015	11,555	15,375	11,025				
Mean		L. 8,447	7,604	8,026	6,662	5,830	4,880	5,580	5,670	4,720	3,723	0·422	0·483	2·82	2·10
		P. 5,286	4,715	5,000	6,260	7,960	8,140	9,210	11,230	10,300	9,587				
		T. 13,733	12,319	13,026	12,922	13,790	13,020	14,790	16,900	15,020	13,310				

TABLE IV.—*Leucocyte Counts in Black and White Rats Before and After Intraperitoneal Injection of 2·5 c.c. Serum from Wistars "Immunized" by Homografts of Skin.*

Number of animal.	Sex.	Pre-injection counts.			Post-injection counts.							L.R. min. Ratio of	L.R. 4.	P.R. max. Ratio of	P.R. 4.
		1 (− ½ hr.).	2 (− 0 hr.).	Mean.	1 (+ ½ hr.).	2 (+ 1 hr.).	3 (+ 1½ hr.).	4 (+ 2 hr.).	5 (+ 2½ hr.).	6 (+ 3 hr.).	7 (+ 4 hr.).				
182	M.	L. 6,300	6,300	6,300	5,400	4,500	3,600	3,600	3,600	3,600	3,600	0·571	0·571	3·43	3·43
		P. 3,600	3,600	3,600	6,300	6,300	6,300	6,300	6,300	6,300	6,300				
217	M.	L. 6,300	6,300	6,300	3,600	3,600	3,600	3,600	3,600	3,600	3,600	0·571	0·571	3·43	3·43
		P. 3,600	3,600	3,600	6,300	6,300	6,300	6,300	6,300	6,300	6,300				
218	M.	L. 6,300	6,300	6,300	3,600	3,600	3,600	3,600	3,600	3,600	3,600	0·571	0·571	3·43	3·43
		P. 3,600	3,600	3,600	6,300	6,300	6,300	6,300	6,300	6,300	6,300				
301	M.	L. 6,300	6,300	6,300	3,600	3,600	3,600	3,600	3,600	3,600	3,600	0·571	0·571	3·43	3·43
		P. 3,600	3,600	3,600	6,300	6,300	6,300	6,300	6,300	6,300	6,300				
303	M.	L. 6,300	6,300	6,300	3,600	3,600	3,600	3,600	3,600	3,600	3,600	0·571	0·571	3·43	3·43
		P. 3,600	3,600	3,600	6,300	6,300	6,300	6,300	6,300	6,300	6,300				
285	F.	L. 6,300	6,300	6,300	3,600	3,600	3,600	3,600	3,600	3,600	3,600	0·571	0·571	3·43	3·43
		P. 3,600	3,600	3,600	6,300	6,300	6,300	6,300	6,300	6,300	6,300				
287	F.	L. 6,300	6,300	6,300	3,600	3,600	3,600	3,600	3,600	3,600	3,600	0·571	0·571	3·43	3·43
		P. 3,600	3,600	3,600	6,300	6,300	6,300	6,300	6,300	6,300	6,300				
289	F.	L. 6,300	6,300	6,300	3,600	3,600	3,600	3,600	3,600	3,600	3,600	0·571	0·571	3·43	3·43
		P. 3,600	3,600	3,600	6,300	6,300	6,300	6,300	6,300	6,300	6,300				
317	F.	L. 6,300	6,300	6,300	3,600	3,600	3,600	3,600	3,600	3,600	3,600	0·571	0·571	3·43	3·43
		P. 3,600	3,600	3,600	6,300	6,300	6,300	6,300	6,300	6,300	6,300				
Mean		L. 6,300	6,300	6,300	3,600	3,600	3,600	3,600	3,600	3,600	3,600	0·571	0·571	3·43	3·43
		P. 3,600	3,600	3,600	6,300	6,300	6,300	6,300	6,300	6,300	6,300				
		T. 9,900	9,900	9,900	9,900	9,900	9,900	9,900	9,900	9,900	9,900				

TABLE IV.—*Leucocyte Counts in Black and White Rats Before and After Intraperitoneal Injection of 2·5 c.c. Serum from Wistars "Immunized" by Homografts of Skin.*

Number of animal.	Sex.		Pre-injection counts. 1 (− ½ hr.).	Pre-injection counts. 2 (− 0 hr.).	Pre-injection counts. Mean.	Post-injection counts. 1 (+ ½ hr.).	Post-injection counts. 2 (+ 1 hr.).	Post-injection counts. 3 (+ 1½ hr.).	Post-injection counts. 4 (+ 2 hr.).	Post-injection counts. 5 (+ 2½ hr.).	Post-injection counts. 6 (+ 3 hr.).	Post-injection counts. 7 (+ 4 hr.).	L.R. min. Ratio of lowest lymphocyte count after injection to mean pre-injection lymphocyte count.	L.R. 4. Ratio of lymphocyte count 4 hours after injection to mean pre-injection lymphocyte count.	P.R. max. Ratio of highest polymorph count after injection to mean pre-injection polymorph count.	P.R. 4. Ratio of polymorph count 4 hours after injection to mean pre-injection polymorph count.
238	M.	L.	3,680	2,565	3,123	2,405	2,130	2,415	1,910	2,780	2,760	2,320	0·612	0·743	4·67	4·23
		P.	2,670	2,110	2,390	2,220	3,470	8,085	10,190	11,170	9,360	10,580				
240	M.	L.	5,695	4,995	5,350	4,980	3,340	2,800	1,585	2,200	2,960	Count	0·297*	Count lost	Count lost	Count lost
		P.	3,490	3,330	3,410	3,320	7,785	10,525	12,690	12,475	10,515	lost				
242	M.	L.	3,645	3,180	3,412	3,040	2,620	2,435	2,480	2,490	1,920	2,100	0·563	0·615	8·35	4·95
		P.	2,430	2,820	2,625	4,500	13,730	21,915	15,220	17,410	12,855	10,300				
310	M.	L.	10,350	8,350	9,350	7,650	6,880	3,960	4,070	3,180	3,470	5,480	0·340	0·586	3·35	3·18
		P.	3,775	4,200	3,988	4,080	8,620	11,570	13,380	10,950	9,080	12,700				
312	M.	L.	15,275	15,860	15,568	14,400	13,580	13,925	11,235	7,700	7,520	5,410	0·347	0·347	4·05	2·15
		P.	6,625	6,640	6,633	11,650	26,845	19,875	20,865	24,225	16,595	12,840				
292	F.	L.	5,760	6,270	6,015	2,820	3,245	2,440	5,580	2,900	2,770	1,780	0·296	0·296	4·40	1·66
		P.	5,950	5,500	5,725	7,140	9,910	13,050	25,160	11,620	9,550	9,710				
294	F.	L.	4,530	4,970	4,750	5,260	2,450	2,070	2,500	3,070	2,020	2,365	0·425	0·498	2·01	1·80
		P.	4,690	4,410	4,550	4,760	7,975	8,930	9,140	7,710	6,450	8,170				
296	F.	L.	3,630	3,950	3,790	3,490	3,905	3,260	3,390	2,145	1,340	1,440	0·354	0·380	1·91	1·35
		P.	2,860	3,640	3,250	3,810	5,090	5,930	6,215	6,160	4,100	4,420				
304	F.	L.	10,420	10,620	10,520	12,990	7,830	5,680	5,920	5,960	3,640	4,550	0·346	0·433	4·35	2·57
		P.	4,180	2,530	3,355	3,790	8,220	11,120	11,760	14,590	10,720	8,900				
314	F.	L.	6,300	5,050	5,675	4,500	3,630	3,830	2,980	4,400	3,480	3,325	0·525	0·586	1·65	1·62
		P.	4,425	3,225	3,825	4,925	6,145	6,045	4,670	5,850	6,295	6,175				
Mean		L.	6,929	6,581	6,755	5,020	4,961	4,282	4,165	4,081	3,133	3,197	0·410	0·498	3·86	2·61
		P.	4,110	3,841	3,975	6,154	9,379	11,705	13,929	12,216	8,960	9,088				
		T.	11,039	10,422	10,730	11,174	14,340	15,987	18,094	16,297	12,093	12,283				

* Whatever the value of L.R.$_4$ which would have been obtained from the lost final count, the value of L.R.min. for this animal could not have exceeded the figure shown.

TABLE V.—*Statistical Analysis of Results by "Student's 't' Test."*

Mean values compared.	"t."	Degrees of freedom.	"P." i.e., probability of result arising as an error of random sampling.	Conclusion.
Mean pre-injection "lymphocyte" counts in Groups I and II	1·62	18	>0·1	No reason to doubt that the animals in all three Groups belong to the same "population."
" " " I and III	0·46	18	>0·6	
Mean pre-injection polymorph counts in Groups I and II	0·45	18	>0·6	
" " " I and III	0·74	18	>0·4	
L.R. min. for Groups I and II	4·93	18	<0·001	Serum from recipients of lymph-node homografts differs significantly from control serum in its anti-lymphocytic effect, but not in its effect on the polymorph count.
L.R.$_4$ for Groups I and II	4·62	18	<0·001	
"Lymphocyte" count 4 hours after injection for Groups I and II	1·90	18	0·08	
P.R. max. for Groups I and II	0·11	18	>0·9	
P.R.$_4$ for Groups I and II	0·35	18	>0·7	
L.R. min. for Groups I and III	4·99	18	<0·001	Serum from recipients of skin homografts differs significantly from control serum in its antilymphocytic effect, but not in its effect on the polymorph count.
L.R.$_4$ for Groups I and III	3·95	17	<0·001	
"Lymphocyte" count 4 hours after injection for Groups I and III	2·38	17	<0·05	
P.R. max. for Groups I and III	1·40	17	>0·15	
P.R.$_4$ for Groups I and III	0·62	17	>0·5	

Note.—Group I comprises black and white rats receiving injection of normal Wistar serum (i.e. Controls).
Group II " " " serum from Wistars which had received a previous homograft of lymph-node.
Group III " " " serum from Wistars which had received a previous homograft of skin.

the fact that the pre-injection counts were slightly (though not significantly) lower in the latter.

In the animals injected with serum from recipients of lymph-node grafts the " lymphocyte " count after 4 hours is also lower than in the controls, but in this case the difference does not quite reach the conventional level of significance.

4. A marked increase in the polymorph count occurred in all three groups, but the figures show no significant difference between the controls and the animals injected with serum from the recipients of grafts.

DISCUSSION.

It is apparent that after a Wistar rat has received a homograph of either lymph-node or skin from a black and white rat, its serum acquires the property of producing an abnormal degree of lymphocytopenia when injected into the donor of the graft.

It would seem that the most reasonable explanation of these findings is that homografts of both lymphoid tissue and skin in the rat give rise to the formation of circulating antibodies, and further, that there exists an antigen common to lymphoid tissue, or lymphocytes, and skin. This hypothesis is consistent with Medawar's more general conclusion that leucocytes and skin possess a common antigen.

It would appear likely, however, that as far as the leucocytes are concerned this common antigen is confined to the lymphocytes, since if it were also present in the polymorphonuclear cells one would have expected that the serum from animals which had received homografts would have caused a relative polymorph leucopenia as well as a lymphocytopenia.

No explanation is offered of the lesser, but quite definite, lymphocytopenia which followed the injection of serum in the control group, nor of the polymorphonuclear leucocytosis which occurred in all groups. The possibility of bacterial contamination being responsible for the latter phenomenon can be excluded, since it occurred in the three cases in which culture of the clot and residual serum had proved sterile. The use of ether anaesthesia was probably partly responsible, but would not seem to provide the full explanation. Despite the known effect of ether in causing a polymorph leucocytosis (Whitby and Britton, 1947), it was considered better to accept this rather than be faced with the uncontrollable fluctuations in leucocyte count which may occur as a result of fear or struggling (Farris, 1938) if samples of blood are taken from rats without anaesthesia.

Whatever the explanation of the change in leucocyte counts in the controls, however, their occurrence would not appear to invalidate our conclusions, which are based on the fact that the lymphocytopenia which occurred after injections of serum from animals which had received homografts was consistently and significantly greater than that which occurred in the controls.

SUMMARY.

By studying the variations in the absolute lymphocyte count of black and white rats following injections of serum from normal Wistars and from Wistars which had received previous homografts of either skin or lymph-node, evidence has been obtained which suggests that such homografts lead to the formation of circulating antibodies. The findings are consistent with the conclusion of

Medawar that there exists an antigen common to leucocytes and skin, and suggest that this antigen is present in the lymphocytes rather than in the polymorphs. A standardized method is described for making leucocyte counts in rats.

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EXPERIMENTAL PEPTIC ULCERATION WITH A NEURO-VASCULAR BASIS.

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REPEATED subcutaneous injections of pilocarpine hydrochloride were given to adult guinea-pigs in experiments designed to study the relationship of Vitamin A metabolism to the pulmonary lesions of fibrocystic disease of the pancreas. Farber (1942) reported the successful reproduction of this condition of the pancreas in kittens by the prolonged administration of parasympatheticomimetic drugs, and he recommended pilocarpine as yielding the best results. No pancreatic lesions were observed in a series of 54 guinea-pigs submitted to this procedure. Injections were given twice daily, commencing with a dose of 0·5 mg., and gradually increasing it to 10·0 mg. as tolerance to the drug was acquired. The course of injections lasted from two to five weeks.

Acute ulceration or haemorrhages into the mucosa of the stomach or duodenum was found in 26 (48 per cent) of the guinea-pigs (Fig. 1), with subsequent perforation and peritonitis in a few cases. Rehfuss (1909) reported the production in animals of such ulcers and haemorrhagic erosions following subcutaneous injections of toxic doses of pilocarpine. Others (e.g. Westphal, 1914 ; Underhill and Freiheit, 1928) have since confirmed this finding. In the present series of animals the capricious anatomical distribution of the lesions, and the " map-like " shape of the larger ones, suggested a vascular basis with the production of infarcts.

Barclay and Bentley (1949), using the technique of microarteriography, deduced the presence of arterio-venous anastomoses in the submucous layer of the human stomach. More recently, Walder (1950) perfused stomachs within

EXPLANATION OF PLATES.

FIG. 1.—Acute gastric ulcers in guinea-pig following course of pilocarpine injections. (× 1½.)
FIG. 2.—Stomach injected with colloidal silver iodide after pilocarpine injection—showing filling defect on the anterior wall. (× 1½.)
FIG. 3.—Stomach and duodenum injected after administration of pilocarpine. Blackening by a photographic developer shows a large defect in the antral region. Note the normal less intense vascularity of the duodenum. (× 1½.)
FIG. 4.—Frozen section of injected normal stomach—42 μ thick. (× 125.)
FIG. 5.—Surface view of injected normal gastric mucosa, showing superficial capillary network. (× 30.)
FIG. 6.—Surface view of injected muscle coat, dissected from the mucosa. (× 60.)
FIG. 7.—Surface view of junction between normal and deficient mucosal filling. Arterio-venous anastomosis indicated by arrow. (× 60.)
FIG. 8.—Transverse section showing edge of filling defect of the mucosa—42 μ thick. (× 60.)
FIG. 9.—Transverse section of edge of ischaemic area—100 μ thick. (× 30.)
FIG. 10.—Transverse section of normal duodenum—42 μ thick. (× 30.)
FIG. 11.—Transverse section of duodenum following pilocarpine—42 μ thick. (× 30.)

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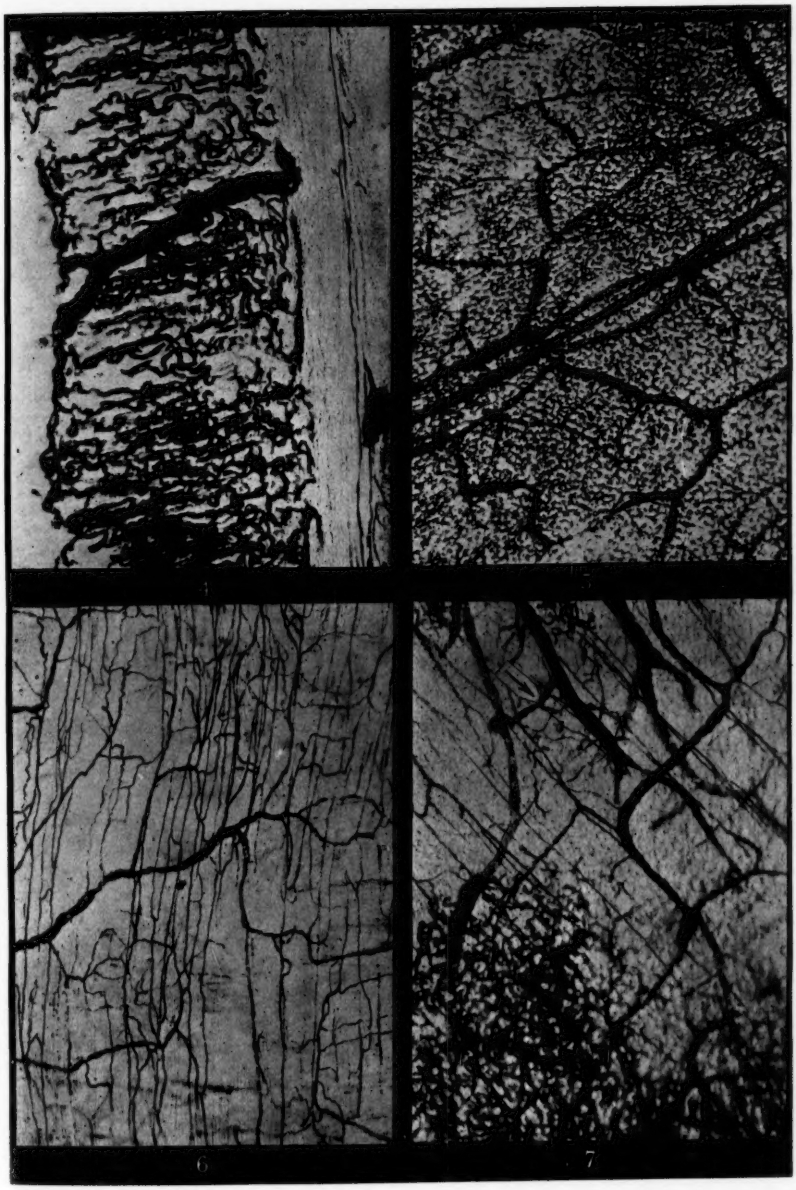

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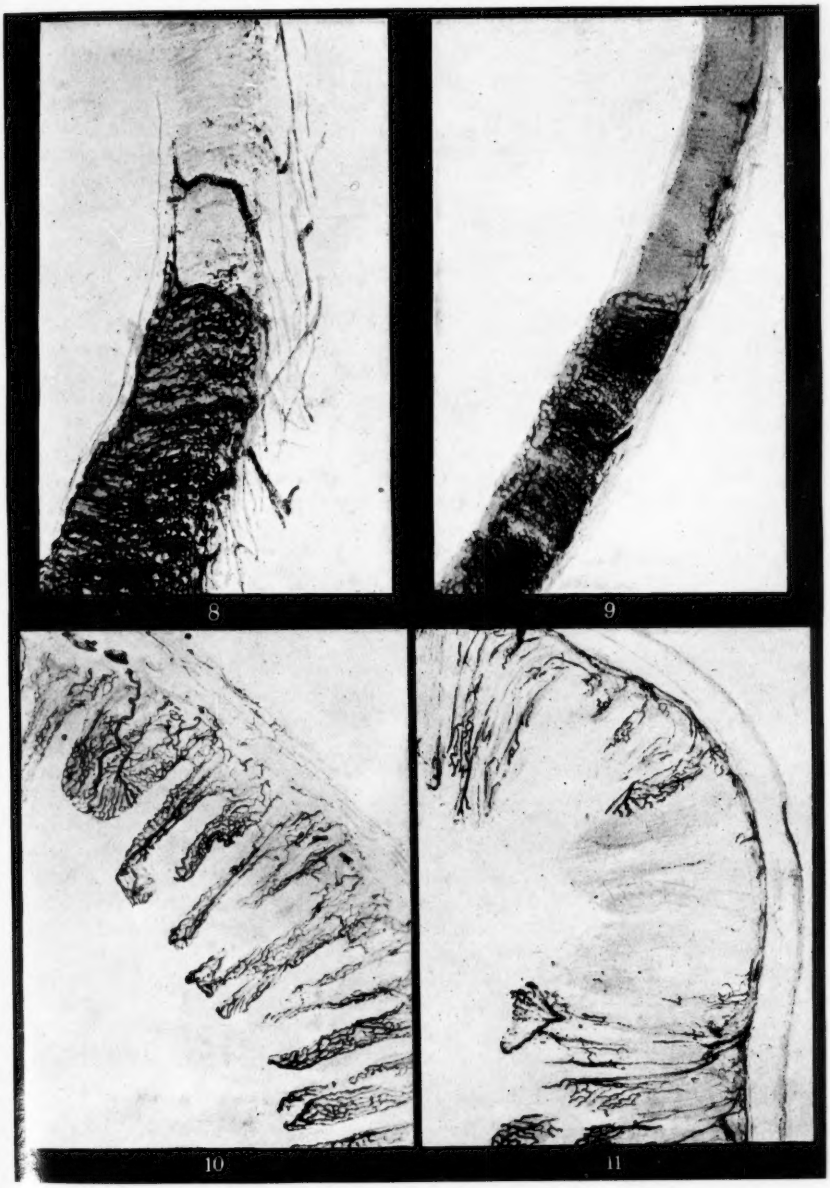

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a few minutes of their operative removal, and found that glass spheres of a mean diameter of 160 μ added to the perfusate entering the right gastro-epiploic artery were recovered in large numbers from the right gastro-epiploic vein, thus confirming the existence of arteriovenous anastomoses.

Vascular dysfunction related to such arterio-venous anastomoses and the deprivation of arterial blood in the capillaries of the gastric mucosa would predispose to peptic ulceration if prolonged. The effect of subcutaneous injections of pilocarpine on the circulation in the stomach wall was therefore investigated.

METHODS.

Healthy, adult guinea-pigs of either sex were given a single subcutaneous injection of from 10 to 20 mg. of pilocarpine hydrochloride into the lower right quadrant of the anterior abdominal wall. They were killed twenty minutes later with coal-gas. The thoracic and abdominal viscera were immediately exposed, and the arterial system of the abdomen was injected with 10 per cent colloidal silver iodide via a cannula inserted into the descending thoracic aorta, using a pressure of 80 mm. of mercury. On completion of the injection the stomach and duodenum were removed and fixed for forty-eight hours in formal-acetate solution (an aqueous solution of 8 per cent formalin and 4 per cent sodium acetate). The silver iodide injection mass was subsequently converted to silver by a photographic developer, thus revealing a black vascular pattern which was studied in frozen sections and in cleared areas of the whole thickness of the stomach wall.

RESULTS.

During the process of intra-arterial injection the white colloidal silver iodide was seen to course along the arteries on the serous surface of the stomach, followed by whitening of areas of the gastric wall. The injected areas enlarged and coalesced, the change being accompanied by filling of the surface veins, which in the main run alongside the arteries. Replacement of the blood in all surface arteries and veins by white injection mass was the criterion for full injection of the gastric circulation.

In 19 out of 20 guinea-pigs the stomach showed sharply defined areas, which remained a dark red colour in contrast to the surrounding white discoloration (Fig 2.). The surface arteries and veins related to these areas had been completely permeated by the injection. The areas were occasionally single, but were more usually multiple, there being one large area and several smaller ones. They presented an irregular shape, and were variable in size and in anatomical distribution. On opening the stomach, after its removal from the animal, the filling defects were found to be much more prominent on the mucosal surface. This observation was facilitated by the addition of a trace of Brilliant Green to the injection fluid. The results obtained macroscopically were therefore strongly suggestive of patchy mucosal ischaemia provoked by the administration of pilocarpine. The relatively thick mucous membrane and thin muscle layer of the guinea-pig stomach would explain the observation of mucosal filling defects when viewed from the outside.

In all of the 20 cases the duodenum appeared to be much less vascular than the stomach, and consequently there was no evidence of mucosal ischaemia in this

region on macroscopical examination. In some instances the whole stomach together with the attached duodenum was opened, and after fixation was treated with D.163 (Kodak) for fifteen minutes. The consequent blackening of injected vessels intensified the difference between the normal and deficient areas of mucosal filling (Fig. 3).

Both normal and ischaemic areas of stomach were taken for microscopical study, but in the case of the duodenum one or two areas were sampled at random.

Transverse sections of normal areas of the stomach show a fairly dense arrangement of tortuous mucosal capillaries which run up between the epithelial tubules to form an anastomosing network just beneath the surface of the mucous membrane. These superficial anastomoses are visible in transverse sections as a prominent line (Fig. 4), but when viewed in cleared portions of whole stomach wall they are seen to constitute a close network which invests the gastric tubules (Fig. 5). No distinct submucous vascular network has been detected in any of the preparations. The arrangement of the vessels in the muscle layer is of a characteristic type and is best seen in the surface views. A few large branches of the surface arteries and veins are present, together with numerous very long vessels which run more or less parallel (Fig. 6). These straight vessels are very fine, and link up with each other at infrequent intervals by short loops. In transverse sections, tinted with a counterstain such as eosin, the long parallel vessels are seen to be travelling along the line of the muscle bundles.

Areas of filling defect confirm that the absence of injection is in the mucosal capillary network (Fig. 7, 8 and 9). In transverse sections the mucous membrane sometimes shows a short extension of the superficial capillary anastomoses into the ischaemic area, and linked with this extension there is a prominent large blood vessel running vertically through the mucosa (Fig. 8). It is considered that such vessels are venules draining the superficial network of anastomoses, because their injection content often shows admixture with blood. The larger vessels of the muscle and peritoneal layers frequently show perfect filling of the artery with less uniform filling of the parallel vein. Beneath the uninjected mucosal capillaries there are a few arterioles and venules running through the submucous layer into the muscle, where they are accompanied by the long straight vessels typical of this coat. Occasionally a short vessel is seen connecting arterial and venous pathways (Fig. 7).

It has already been mentioned that the examination of the duodenum was limited to a few haphazard sections. The duodenum has a vascular pattern comparable to that of the stomach (Fig. 10), but by virtue of its villous nature the mucous membrane is not so compact, and hence appears less vascular macroscopically. In three of the twenty animals, some sections of the duodenum revealed absent filling of two or three epithelial processes, but with an intact circulation in the underlying submucous and muscle layers (Fig. 11).

CONTROLS.

Six guinea-pigs were not injected with pilocarpine, but were sacrificed with coal-gas and submitted to the same procedure for demonstrating the vascular network. In none of these animals was any defect in filling of the mucosal vessels of the stomach or duodenum detected. Any effect resulting from carbon monoxide asphyxia was thus excluded as a factor in the production of mucosal ischaemia.

A further 6 animals were injected subcutaneously with normal saline. The injection was of the same volume (viz. 2 ml.) as that administered to the pilocarpine series, and was given in the same site. After the lapse of twenty minutes they were killed with coal-gas and investigated by the same technique as the other two series. Again, no evidence of mucosal ischaemia was found in these controls, thus ruling out the possibility of the trauma of injection causing a reflex disturbance of the circulation in the gastric or duodenal mucosa. It is worthy of note that no obvious difference between the pilocarpine and control series was detected in the submucous and muscle layers.

DISCUSSION.

The action of the vagus nerves has figured greatly in both experimental and clinical studies of peptic ulceration. Pilocarpine injected subcutaneously may exert its influence on the stomach either directly via the blood stream or secondarily by central nervous stimulation. At the instigation of Cushing (1931) it was shown by Light, Bishop and Kendall (1932) that gastric erosions could be produced in rabbits by injecting small doses of pilocarpine into the cerebral ventricles, whereas much larger doses were required to elicit comparable effects when the injection was given by the subcutaneous route. In both cases it would seem that the mucosal ischaemia is due to vagal action. The ischaemia is patchy in distribution, and thus corresponds to the disposition of experimental peptic ulcers produced by pilocarpine injections.

It is obvious that one must consider the role of arteriovenous anastomoses in the mechanism of this ischaemia, and also the influence of spasm of gastric musculature. These relationships cannot be satisfactorily investigated by purely static morphological studies of the dead tissues, and experiments now in progress have been designed to assess such influences from a more physiological aspect.

In the light of the work by Barclay and Bentley (1949) it is tempting to infer that arteriovenous anastomoses play a part in the mucosal vascular dysfunction occasioned by the administration of pilocarpine, but even if this inference is permissible the role of anastomosis must surely be of a secondary or minor character : mere shunting of arterial blood via arterio-venous anastomoses will not result in total deprivation of the mucosa unless there is an associated spasm which prevents capillary filling.

SUMMARY.

The effect of a subcutaneous injection of a large dose of pilocarpine hydrochloride on the circulation in the stomach and duodenum has been investigated in guinea-pigs by studies of the injected blood vessels.

In 19 out of 20 animals the injection resulted in patchy ischaemia of the mucosa of the stomach, whilst in three of the cases a similar effect was observed in the duodenum.

Control experiments show that the phenomenon is due entirely to the administration of pilocarpine, and indicate autonomic imbalance as the causal factor. Possible mechanisms of parasympathetic stimulation are mentioned.

No evidence could be demonstrated by the technique employed that arteriovenous anastomoses are concerned in the production of the ischaemia.

The ischaemic lesions would predispose to peptic ulceration, and their anatomical distribution and morphology are strictly comparable to the ulcers and haemorrhagic erosions observed in guinea-pigs submitted to a course of repeated subcutaneous injections of pilocarpine.

It is probable that the experimental ulcers produced by a wide variety of methods based on autonomic imbalance are the result of initial mucosal ischaemia.

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FACTORS INFLUENCING THE AGGLUTINABILITY OF RED CELLS: THE DEMONSTRATION OF A VARIATION IN THE SUSCEPTIBILITY TO AGGLUTINATION EXHIBITED BY THE RED CELLS OF INDIVIDUAL OXEN.

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DURING a series of experiments in which the anti-globulin sensitization test (Coombs, Mourant and Race, 1945) was being used to detect antibody sensitization of the red cells of different animals, sensitized ox cells were observed to exhibit a wide variation in their susceptibility to agglutination. Cells from certain oxen, in fact, were found to be inagglutinable by this technique.

Previously, Bailey and Raffel (1935), in a study of the haemolytic property of infectious mononucleosis sera, had observed that although ox red cells were fully susceptible to lysis in the presence of guinea-pig complement, they were highly resistant to agglutination by these sera. They also showed that ox red cells possessed, in common with sheep red cells, a heat-stable antigen with which these antibodies could be absorbed.

From their observations it seemed reasonable to postulate that, although ox red cells treated with infectious mononucleosis sera were not agglutinated, they were probably sensitized and therefore should be agglutinated by the anti-globulin sensitization technique used for the demonstration of the sensitization of human red cells by "incomplete" Rh antibodies.

Ox red cells, therefore, were treated with a number of different infectious mononucleosis sera, but no agglutination was obtained with highly potent rabbit anti-human globulin sera.

This failure of rabbit anti-human globulin sera to detect the presence of the antibody in infectious mononucleosis sera when adsorbed on ox cells raised doubts as to whether this agent, although responsible for the Paul-Bunnell reaction, was, in fact, an antibody globulin. However, the fractionation of infectious mononucleosis sera by salting-out methods showed that the haemolytic and agglutinating properties of these sera for ox and sheep cells respectively followed closely the distribution of the α and β iso-antibodies. Furthermore, filtration through a gradocol membrane of pore diameter 67 m.μ did not remove these properties. These observations lent no support to the suggestion that the agent responsible for the Paul-Bunnell reaction was not an antibody globulin.

In a further series of experiments, ox red cells treated with infectious mononucleosis sera were examined by the bovine albumen (Diamond and Denton,

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1945) and the cholera filtrate techniques (Pickles, 1946), but again the treated cells could not be made to agglutinate.

Attempts were also made to detect the sensitization of ox red cells by infectious mononucleosis sera using not only rabbit and guinea-pig anti-human globulin sera but also special anti-globulin sera produced by immunizing rabbits, guinea-pigs and oxen with washed sensitized ox cells carrying the Paul-Bunnell antibody adsorbed *in situ*. These latter sera were absorbed free of antibodies to washed normal ox cells before being used in these tests. Although some degree of agglutination was observed, the results as a whole, in contrast to those obtained with a human red-cell " incomplete " Rh-antibody control system, were extremely inconsistent.

At this point in the investigation the possibility that some hitherto unsuspected variation in the constitution of the ox red cells might be responsible for the inconsistency of these results was considered.

To investigate this, samples of red cells from five different oxen were examined simultaneously, after a previous treatment with varying dilutions of an infectious mononucleosis serum, by the rabbit anti-human globulin technique. The results showed that ox red cells exhibit a wide range of susceptibility to agglutination. Furthermore, the particular cells which had been used for all the previous experiments were found to be the most resistant of all to agglutination.

Further investigation showed that this variation in the agglutinability of ox cells was not confined to this system. Cell samples from the same oxen treated with rabbit or guinea-pig anti-ox red cells sera behaved in an exactly similar manner.

It was also of interest to find that ox cells classed as highly resistant to agglutination by these strictly serological techniques were, however, strongly clumped by the trypsin method of detecting antibody sensitization (Morton and Pickles, 1947). On the other hand, no agglutination of any of these ox cell suspensions, whether sensitized with infectious mononucleosis sera or homologous rabbit antisera, was obtained with the bovine albumen technique,

We have now examined red cell samples from sixteen oxen in all, and find that these cells fall into three broad classes, namely " markedly " and " moderately agglutinable " and " inagglutinable." For convenience in presentation, the experimental data which follow have been mainly confined to observations on samples of red cells from three oxen, each a representative of one of these three broad classes of agglutinability.

MATERIALS.

Sheep and ox red cells.

Bi-weekly samples were obtained by venipuncture, defibrinated with glass beads and stored at 4° C. When required for use, the cells were washed three times in saline and the appropriate suspensions made from the packed cell deposit.

Sera.

(a) *Infectious mononucleosis sera.*—Sera were obtained from cases of infectious mononucleosis giving strongly positive Paul-Bunnell reactions and the characteristic absorption pattern with boiled ox cell and guinea-pig kidney suspensions.

(b) *Guinea-pig anti-ox red cell sera.*—Six guinea-pigs each received five intraperitoneal inoculations of 1·0 ml. of a 10 per cent suspension of washed ox red cells at three-day intervals, and were bled by cardiac puncture on the 9th day after the last injection. Guinea-pigs 1980 and 1984 received cells from ox 5153; 1967 and 1968 from ox 5156; and 1977 and 1978 from ox 5157.

(c) *Rabbit anti-ox red cell sera.*—Six rabbits each received five intravenous injections of 1·0 ml. of a 10 per cent suspension of washed ox red cells at three-day intervals, and were bled from the ear vein on the 9th day following the last inoculation. Rabbits 1876 and 1669 received cells from ox 5153; 1789 and 1823 from ox 5156; and 1821 and 1840 from ox 5157.

All these sera were inactivated at 56° C. for half an hour before use.

(d) *Sera for anti-globulin sensitization tests.*—The rabbit anti-human globulin sera were prepared as described previously (Coombs and Mourant, 1947). The rabbit anti-guinea-pig globulin serum was prepared in a similar manner. None of these rabbit antisera possessed agglutinins for ox cells, so no absorption was required for these tests. The anti-rabbit-globulin serum was produced in a goat by inoculations of whole rabbit serum. This anti-globulin serum was absorbed with well washed normal ox cells before being used in these experiments.

Complement.

Unheated guinea-pig serum was the source of haemolytic complement used in these tests. No normal haemolysin activity for ox cells was observed at the dilutions used (2–4 M.C.D.).

Trypsin solution.

A 2 per cent solution of British Drug Houses' powdered trypsin preparation was made in M/15 phosphate buffer pH 7·2. This was clarified by centrifugation and stored at — 20° C.

METHODS.

Test for haemolysin activity.

A two-fold serial dilution of the serum was made in 0·1 ml. amounts of saline, and 0·1 ml. of a 1 per cent red cell suspension and 0·1 ml. guinea-pig complement (2–4 M.C.D.) added to each tube. After incubation for half an hour in the 37° C. water-bath, the tubes were centrifuged and the degree of haemolysis read.

On some occasions the test was carried out in two stages. The cells were first added to the serum dilutions and the tubes incubated for half an hour in the 37° C. water-bath. The sensitized cells were then washed in saline before adding the complement. After incubation for a further half hour, the tubes were centrifuged and the degree of haemolysis read.

Both methods gave the same results.

Test for agglutinin activity.

A two-fold dilution of the serum was made in 0·1 ml. amounts of saline and 0·1 ml. of a 1 per cent suspension of red cells added to each tube. After incubation for 1½ hours at 37° C. followed by a light centrifugation for 30 seconds, the cell deposits were pipetted on to glass slides and read under the microscope.

Anti-globulin sensitization test (indirect method).

A two-fold dilution of the serum was made in 0·1 ml. amounts of saline and 0·1 ml. of a 1 per cent suspension of red cells added to each tube. After incubation for 1 hour at 37° C., the tubes were lightly centrifuged and the supernatants discarded. The cell deposits were then washed twice in saline and resuspended in 0·1 ml. amounts of saline. Single drops from each tube were transferred to duplicate sets of small precipitin tubes (Rh agglutination tubes). To each tube of one set a single drop of a suitable dilution of the anti-globulin serum was added. The same volume of a corresponding dilution of normal serum or of saline was added to each tube of the second set.

All tests were then reincubated for 1½ hours at 37° C. After a light centrifugation for 30 seconds, the cell deposits were pipetted on to glass slides and read under the microscope.

For cells treated in the first place with infectious mononucleosis sera, rabbit anti-human globulin sera were used. For the detection of sensitization by rabbit and guinea-pig anti-ox sera, a goat anti-rabbit globulin and a rabbit anti-guinea-pig globulin serum was employed, respectively.

Trypsin method of detecting sensitization.

These tests were carried out in an exactly similar manner to the anti-globulin sensitization tests, except that a 1 per cent solution of trypsin was substituted for the anti-globulin serum. This solution was prepared by diluting the 2 per cent stock solution with an equal volume of saline immediately before use.

Diamond's bovine albumen agglutination test.

Except for the substitution of a 20 per cent solution of Armour's bovine albumen for saline as the suspending medium, these tests were carried out in an exactly similar manner to the ordinary agglutination tests.

EXPERIMENTAL STUDY.

The sensitization of ox red cells by infectious mononucleosis sera as demonstrated by immune haemolysis, agglutination and the anti-globulin sensitization technique.

Six infectious mononucleosis sera were titrated against ox cells 5153, 5156 and 5157. Evidence of antibody combination was sought for by agglutination and by the antiglobulin sensitization technique using a rabbit anti-human globulin serum.

From the results (Table I) it may be seen that although a certain degree of direct agglutination of cells 5153, and to a less extent of cells 5156, was observed, cells 5157 in no case showed agglutination.

Three grades of response were obtained by the antiglobulin sensitization technique with each of the six infectious mononucleosis sera. Cells 5153 were markedly agglutinable, cells 5156 appreciably less so, and cells 5157 again remained inagglutinable throughout.

On the other hand, all three cell samples showed an equal susceptibility to lysis in the presence of guinea-pig complement. Further evidence that the same

TABLE I.—*Results of Tests to Demonstrate the Sensitization of Ox Red Cells 5153, 5156 and 5157 with the Paul-Bunnell Antibody.*

Infectious mono-	Ox	Immune haemolysis. Serum dilutions.	Direct agglutination. Serum dilutions.	Anti-globulin sensitization test. Serum dilutions.

TABLE I.—*Results of Tests to Demonstrate the Sensitization of Ox Red Cells* 5153, 5156 *and* 5157 *with the Paul-Bunnell Antibody.*

Infectious mononucleosis sera.	Ox cells.	Immune haemolysis. Serum dilutions. 1/5.	1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.	1/1280.	Saline.	Direct agglutination. Serum dilutions. 1/5.	1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.	1/1280.	Saline.	Anti-globulin sensitization test. Serum dilutions. 1/5.	1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.	1/1280.	Saline.
Co.	5153	H	H	H	H	H	H	H	H	H	–	+	+	–	–	–	–	–	–	–	–	++	++	++	++	++	++	++	+	–	–
	5156	H	H	H	H	H	H	H	H	H	–	–	–	–	–	–	–	–	–	–	–	++	++	++	++	+	+	–	–	–	–
	5157	H	H	H	H	H	H	H	H	H	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Sm.	5153	H	H	H	H	H	H	H	H	H	–	++	++	++	++	w	–	–	–	–	–	++	++	++	++	++	++	+	w	–	–
	5156	H	H	H	H	H	H	H	H	H	–	++	+	w	–	–	–	–	–	–	–	++	++	++	++	+	w	–	–	–	–
	5157	H	H	H	H	H	H	H	H	H	–	–	–	–	–	–	–	–	–	–	–	++	++	w	–	–	–	–	–	–	–
Tu.	5153	H	H	H	H	H	H	H	H	–	–	++	++	++	++	++	+	–	–	–	–	++	++	++	++	++	++	++	w	–	–
	5156	H	H	H	H	H	H	H	H	–	–	++	w	–	–	–	–	–	–	–	–	++	++	++	++	w	–	–	–	–	–
	5157	H	H	H	H	H	H	H	H	–	–	–	–	–	–	–	–	–	–	–	–	+	w	–	–	–	–	–	–	–	–
Ba.	5153	H	H	H	H	H	H	H	H	h	–	++	++	++	+	w	–	–	–	–	–	++	++	++	++	++	w	–	–	–	–
	5156	H	H	H	H	H	H	H	H	h	–	–	–	–	–	–	–	–	–	–	–	++	++	++	w	–	–	–	–	–	–
	5157	H	H	H	H	H	H	H	H	h	–	–	–	–	–	–	–	–	–	–	–	++	w	–	–	–	–	–	–	–	–
Sa.	5153	H	H	H	H	H	H	H	h	–	–	++	++	++	++	w	–	–	–	–	–	++	++	++	++	++	+	–	–	–	–
	5156	H	H	H	H	H	H	H	h	–	–	w	–	–	–	–	–	–	–	–	–	++	++	+	w	–	–	–	–	–	–
	5157	H	H	H	H	H	H	H	h	–	–	–	–	–	–	–	–	–	–	–	–	+	w	w	–	–	–	–	–	–	–
Cm.	5153	H	H	H	H	H	H	H	H	h	–	++	++	++	+	w	–	–	–	–	–	++	++	++	++	++	++	+	–	–	–
	5156	H	H	H	H	H	H	H	H	h	–	w	–	–	–	–	–	–	–	–	–	++	++	++	w	–	–	–	–	–	–
	5157	H	H	H	H	H	H	H	H	h	–	–	–	–	–	–	–	–	–	–	–	w	–	–	–	–	–	–	–	–	–

H = complete haemolysis; h = slight haemolysis; – = no haemolysis; ++ = strong agglutination + = agglutination; w = trace agglutination; – = no agglutination.

TABLE II.—*Results of Tests to Demonstrate the Sensitization of Ox Red Cells* 5153, 5156 *and* 5157 *with Guinea-pig Anti-ox Red Cell Sera.*

Guinea-pig anti-ox red cell sera.	Ox cells.	Immune haemolysis. Serum dilutions. 1/5.	1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.	1/1280.	Saline.	Direct agglutination. Serum dilutions. 1/5.	1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.	1/1280.	Saline.	Anti-globulin sensitization test. Serum dilutions. 1/5.	1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.	1/1280.	Saline.
G-p 1980 anti-ox 5153	5153	H	H	H	H	H	H	H	h	h	–	w	–	–	–	–	–	–	–	–	–	++	++	++	++	++	++	++	++	+	–
	5156	H	H	H	H	H	H	H	h	h	–	–	–	–	–	–	–	–	–	–	–	++	++	++	++	++	w	w	w	–	–
	5157	H	H	H	H	H	H	H	h	h	–	–	–	–	–	–	–	–	–	–	–	+	w	–	–	–	–	–	–	–	–
G-p 1984 anti-ox 5153	5153	H	H	H	H	H	H	H	h	h	–	w	–	–	–	–	–	–	–	–	–	++	++	++	++	++	++	++	++	++	–
	5156	H	H	H	H	H	H	H	h	h	–	–	–	–	–	–	–	–	–	–	–	++	++	++	++	++	+	w	–	–	–
	5157	H	H	H	H	H	H	H	h	h	–	–	–	–	–	–	–	–	–	–	–	+	++	++	++	w	–	–	–	–	–
G-p 1967 anti-ox 5156	5153	H	H	H	H	H	H	H	h	h	–	w	–	–	–	–	–	–	–	–	–	++	++	++	++	++	++	++	++	++	–
	5156	H	H	H	H	H	H	H	h	h	–	–	–	–	–	–	–	–	–	–	–	++	++	++	++	w	w	–	–	–	–
	5157	H	H	H	H	H	H	H	h	h	–	–	–	–	–	–	–	–	–	–	–	–	–	w	w	w	–	–	–	–	–
G-p 1968 anti-ox 5156	5153	H	H	H	H	H	H	H	H	h	–	w	–	–	–	–	–	–	–	–	–	++	++	++	++	++	++	++	++	++	–
	5156	H	H	H	H	H	H	H	H	h	–	–	–	–	–	–	–	–	–	–	–	++	++	++	++	+	w	–	–	–	–
	5157	H	H	H	H	H	H	H	H	h	–	–	–	–	–	–	–	–	–	–	–	–	w	w	w	w	–	–	–	–	–
G-p 1977 anti-ox 5157	5153	H	H	H	H	H	H	H	h	h	–	++	w	–	–	–	–	–	–	–	–	++	++	++	++	++	++	++	++	+	–
	5156	H	H	H	H	H	H	H	h	h	–	+	–	–	–	–	–	–	–	–	–	++	++	++	++	++	w	–	–	–	–
	5157	H	H	H	H	H	H	H	h	h	–	–	–	–	–	–	–	–	–	–	–	+	w	w	–	–	–	–	–	–	–
G-p 1978 anti-ox 5157	5153	H	H	H	H	H	H	H	H	h	–	+	w	–	–	–	–	–	–	–	–	++	++	++	++	++	++	++	++	++	–
	5156	H	H	H	H	H	H	H	H	h	–	–	–	–	–	–	–	–	–	–	–	++	++	++	++	+	+	w	–	–	–
	5157	H	H	H	H	H	H	H	H	h	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	w	w	–	–	–	–

H = complete haemolysis; h = slight haemolysis; – = no haemolysis; ++ = strong agglutination; + = agglutination; w = trace agglutination; – = no agglutination.

amount of Paul-Bunnell antibody was taken up by these different cell samples was provided by absorption tests in which cells 5153 and 5157 were shown to be equally capable of removing the haemolytic and agglutinating properties for sheep red cells from infectious mononucleosis sera.

The sensitization of ox cells by guinea-pig anti-ox red cell sera as demonstrated by immune haemolysis, agglutination and the anti-globulin sensitization technique.

The six guinea-pig anti-ox red cell sera (see " MATERIALS ") were titrated against ox cells 5153, 5156 and 5157. The titres of haemolysin activity in the presence of guinea-pig complement, of direct agglutination and of sensitization as demonstrated by the anti-globulin sensitization technique using a rabbit anti-guinea-pig globulin serum are shown in Table II. The results were very similar to those obtained in the previous experiment.

The sensitization of ox red cells by rabbit anti-ox red cells sera as demonstrated by immune haemolysis, agglutination and the anti-globulin sensitization technique.

Except for the substitution of six rabbit anti-ox red cell sera for the guinea-pig anti-ox red cell sera, and a goat anti-rabbit globulin serum for the rabbit anti-guinea-pig globulin serum in the anti-globulin sensitization tests, these experiments were carried out in a similar manner to the previous experiments. The results shown in Table III, again conform with those of the two previous experiments.

Diamond's bovine albumen agglutination test.

One infectious mononucleosis serum (Co) and one rabbit anti-ox red cell serum (1876) were titrated against ox cells 5153 and 5157 in a 20 per cent bovine albumen medium. No agglutination was obtained with either sample of cells, and even the slight direct agglutination of cells 5153 observed in the previous experiments was inhibited under these conditions.

The demonstration of antibody sensitization of the "inagglutinable" ox cells (5157) *by trypsin.*

Two infectious mononucleosis sera, two rabbit anti-ox red cell sera and two guinea-pig anti-ox red cell sera were titrated against ox cells 5153 and 5157 and examined in parallel by the anti-globulin and trypsin technique for evidence of sensitization. In each case, samples of the washed sensitized cells were resuspended in saline also, to control the degree of agglutination due to antibody sensitization alone.

From the results (Table IV) it may be seen that the degree of sensitization of cells 5153 detected by both techniques was approximately the same. The sensitized cells of 5157, however, although inagglutinable with the anti-globulin technique, were clumped very strongly by the trypsin method.

The slight discrepancy between the degree of agglutination of the sensitized ox cells 5153 observed after washing and resuspension in saline and the results obtained in the direct agglutination tests of the previous experiments was probably due to the difference in the two procedures.

TABLE III.—*Results of Tests to Demonstrate the Sensitization of Ox Red Cells 5153, 5156 and 5157 with Rabbit Anti-ox Red Cell Sera.*

Rabbit anti-ox	Ox	Immune haemolysis. Serum dilutions.	Direct agglutination. Serum dilutions.	Anti-globulin sensitization test. Serum dilutions.

TABLE III.—*Results of Tests to Demonstrate the Sensitization of Ox Red Cells 5153, 5156 and 5157 with Rabbit Anti-ox Red Cell Sera.*

Rabbit anti-ox red cell sera.	Ox cells.	Immune haemolysis. Serum dilutions. 1/5.	1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.	1/1280.	Saline.	Direct agglutination. Serum dilutions. 1/5.	1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.	1/1280.	Saline.	Anti-globulin sensitization test. Serum dilutions. 1/5.	1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.	1/1280.	Saline.
R1876 anti-ox	5153	H	H	H	H	H	H	H	h	h	–	++	++	++	+	–	–	–	–	–	–	++	++	++	++	++	++	+	w	–	–
	5156	H	H	H	H	H	H	H	h	h	–	–	–	–	–	–	–	–	–	–	–	++	+	–	–	–	–	–	–	–	–
	5157	H	H	H	H	H	H	H	h	h	–	–	–	–	–	–	–	–	–	–	–	w	–	–	–	–	–	–	–	–	–
R1669 anti-ox	5153	H	H	H	H	H	H	h	h	–	–	++	++	+	–	–	–	–	–	–	–	++	++	++	++	++	++	++	++	w	–
	5156	H	H	H	H	H	H	h	h	–	–	w	–	–	–	–	–	–	–	–	–	++	++	+	–	–	–	–	–	–	–
	5157	H	H	H	H	H	H	h	h	–	–	–	–	–	–	–	–	–	–	–	–	w	–	–	–	–	–	–	–	–	–
R1789 anti-ox	5153	H	H	H	H	H	H	H	h	–	–	++	++	++	++	w	–	–	–	–	–	++	++	++	++	++	++	++	++	++	–
	5156	H	H	H	H	H	H	H	h	–	–	–	–	–	–	–	–	–	–	–	–	+	w	–	–	–	–	–	–	–	–
	5157	H	H	H	H	H	H	H	h	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
R1823 anti-ox	5153	H	H	H	H	H	H	H	h	–	–	++	++	++	+	–	–	–	–	–	–	++	++	++	++	++	++	++	++	+	–
	5156	H	H	H	H	H	H	H	h	–	–	++	+	w	–	–	–	–	–	–	–	++	++	++	w	–	–	–	–	–	–
	5157	H	H	H	H	H	H	H	h	–	–	+	w	–	–	–	–	–	–	–	–	w	–	–	–	–	–	–	–	–	–
R1821 anti-ox	5153	H	H	H	H	H	H	H	h	–	–	++	++	+	w	–	–	–	–	–	–	++	++	++	++	++	++	++	++	+	–
	5156	H	H	H	H	H	H	H	h	–	–	++	++	w	–	–	–	–	–	–	–	++	++	++	w	w	–	–	–	–	–
	5157	H	H	H	H	H	H	H	h	–	–	+	w	–	–	–	–	–	–	–	–	++	++	w	–	–	–	–	–	–	–
R1840 anti-ox	5153	H	H	H	H	H	H	H	h	h	–	++	++	+	–	–	–	–	–	–	–	++	++	++	++	++	++	+	+	–	–
	5156	H	H	H	H	H	H	H	h	h	–	++	+	–	–	–	–	–	–	–	–	++	++	+	w	–	–	–	–	–	–
	5157	H	H	H	H	H	H	H	h	h	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–

H = complete haemolysis; h = slight haemolysis; – = no haemolysis; ++ = strong agglutination; + = agglutination; w = trace agglutination; – = no agglutination.

TABLE IV.—*A Comparison of Trypsin and Anti-globulin Sera in the Detection of Antibody Sensitization of the "Markedly Agglutinable" and "Inagglutinable" Class of Ox Cell.*

Sensitizing anti-serum.	Sensitized cells treated with:	"Markedly agglutinable": Ox cells 5153. Dilutions of sensitizing serum. 1/5.	1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.	1/1280.	Saline.	"Inagglutinable": Ox cells 5157. Dilutions of sensitizing serum. 1/5.	1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.	1/1280.	Saline.
Infect. mononucleosis serum Co	Saline	w	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Rabbit anti-human glob. serum	++	++	++	++	++	++	++	+	–	–	w	w	–	–	–	–	–	–	–	–
	Trypsin	++	++	++	++	++	++	++	++	–	–	++	++	++	++	++	++	++	++	++	–
Infect. mononucleosis serum Cm.	Saline	++	++	++	w	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Rabbit anti-human glob. serum	++	++	++	++	++	++	++	+	w	–	–	–	–	–	–	–	–	–	–	–
	Trypsin	++	++	++	++	++	++	++	++	++	–	++	++	++	++	++	++	++	++	++	–
Rabbit 1876 anti-ox 5153 serum	Saline	w	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Goat anti-rabbit glob. serum	++	++	++	++	++	++	+	–	–	–	w	–	–	–	–	–	–	–	–	–
	Trypsin	++	++	++	++	++	++	–	–	–	–	++	++	++	++	++	++	++	++	–	–
Rabbit 1840 anti-ox 5157 serum	Saline	++	++	++	–	–	–	–	–	–	–	w	–	–	–	–	–	–	–	–	–
	Goat anti-rabbit glob. serum	++	++	++	++	++	++	+	–	–	–	+	w	–	–	–	–	–	–	–	–
	Trypsin	++	++	++	++	w	–	–	–	–	–	++	++	++	++	++	++	++	++	++	–
Guinea-pig 1980 anti-ox 5153 serum	Saline	++	++	++	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Rabbit anti-guinea-pig glob. serum	++	++	++	++	++	++	++	++	+	–	+	+	w	–	–	–	–	–	–	–
	Trypsin	++	++	++	++	++	++	++	++	+	–	++	++	++	++	++	++	++	++	++	–
Guinea-pig 1977 anti-ox 5157 serum	Saline	+	w	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Rabbit anti-guinea-pig glob. serum	++	++	++	++	++	++	++	++	+	–	++	w	w	w	–	–	–	–	–	–
	Trypsin	++	++	++	++	++	++	w	–	–	–	++	++	++	++	++	++	++	++	++	–

++ = strong agglutination; + = agglutination; w = trace agglutination; – = no agglutination.

Experiment to show that the guinea-pig and rabbit anti-ox cell sera possessed antibodies for ox cells of a different specificity to that of the Paul-Bunnell antibody.

Infectious mononucleosis sera and guinea-pig and rabbit anti-ox red cell sera, before and after absorption with a boiled ox red cell suspension, were tested for their capacity for sensitizing ox cells 5153 and 5157 using the anti-globulin and trypsin techniques.

The boiled ox cell suspension removed all the sensitizing antibody from the infectious mononucleosis sera but did not reduce the titre of sensitizing antibodies in the guinea-pig or rabbit antisera.

It was thus clear that the guinea-pig and rabbit antisera contained antibodies against ox cells of a different specificity to that of the Paul-Bunnell antibody. The inagglutinability of cells 5157 was not confined therefore to reactions involving one particular antigen-antibody system.

Examination of the cells of sixteen oxen to obtain an indication of the frequency of the three broad classes of cell agglutinability.

The washed cells of sixteen oxen were incubated for 1 hour at 37° C. with a guinea-pig anti-ox red cell serum at dilutions of 1 : 10, 1 : 40 and 1 : 320. After sensitization the cells were centrifuged down and washed twice in saline.

Volumes of each of the washed sensitized cell samples were then mixed either with equal volumes of a suitable dilution of a rabbit anti-guinea-pig globulin serum or equal volumes of a 1 per cent trypsin solution. After incubation at 37° C. for 1½ hours, the results were read in the usual manner.

From the results obtained with the anti-globulin serum (Table V) it may be seen that the cells of four oxen fell into the " markedly agglutinable," seven into the " moderately agglutinable," and five into the " inagglutinable " class. On the other hand, trypsin, by its clumping action, detected the sensitization of all the cells. It was, however, interesting to find that the antiglobulin serum was more sensitive than trypsin in detecting the sensitization of the " markedly agglutinable " class of cell.

DISCUSSION.

The experiments reported in this paper demonstrate that ox red cells sensitized with antibodies show a wide variation in their susceptibility to agglutination by standard serological techniques, and that this variation is apparently dependent upon the presence or absence of some factor in the cell itself, irrespective of the antibody employed for sensitization. Of the sixteen oxen studied, the cells of five were " inagglutinable," seven " moderately agglutinable," while the cells of the remaining four were " markedly agglutinable " when tested for sensitization by the anti-globulin sensitization technique. Furthermore, only the cells in the " markedly agglutinable " class showed any appreciable degree of agglutination in the direct agglutination reactions.

Haemolysin and absorption tests clearly showed that the cells of the three classes were equally sensitized by treatment with the various sera employed. This supports the view that this variation in susceptibility to agglutination is due to a property of the cell itself. This phenomenon is thus distinct from the inability of " incomplete " Rh antibodies to bring about agglutination, as in the

TABLE V.—*Examination of Sensitized Red Cells of Sixteen Oxen by the Anti-globulin Serum and Trypsin Methods, and the Classification of their Cells into Three Broad Groups as Regards Their Agglutinability.*

				Technique used to detect sensitization					
				Anti-globulin technique.			Trypsin technique.		
Preliminary sensitization with guinea-pig anti-ox cell sera diluted:	*Bovine.*	*Breed.*	*Age.*	1/10.	1/40.	1/320.	1/10.	1/40.	1/320.
"Markedly agglutinable"	5153	Ayrshire	5½ years	++	++	++	++	++	—
	C1	Jersey	Calf	++	++	++	++	++	−
	C2	Jersey	Calf	++	++	++	++	+	−
	Patsy	Friesian	?	++	++	++	++	++	−
"Moderately agglutinable"	5156	Ayshire shorthorn	3½ years	++	+	−	++	++	++
	C4	Jersey	Calf	++	++	−	++	++	++
	Fancy	Friesian	4 years	+	+	−	++	++	+
	7	„	9 „	++	++	−	++	++	++
	Caroline	„	?	++	++	−	++	++	−
	C3	Jersey	Calf	++	++	−	++	++	++
	Neo	Friesian	13 years	++	++	−	++	++	++
"Inagglutinable"	5157	„	3½ „	−	−	−	++	++	++
	Pansy	„	7 „	w	−	−	++	++	++
	Monica	„	3 „	−	−	−	++	++	++
	Julia	„	3 „	−	−	−	++	++	−
	Lodger	Ayrshire shorthorn	5 „	w	w	−	++	++	++

++ = strong agglutination; + = agglutination; w = trace agglutination; − = no agglutination.

latter case the failure to obtain agglutination is due to the nature of the antibody and not the cell.

Again, the failure to obtain agglutination of the " inagglutinable " cells was not confined to the reactions involving the Paul-Bunnell antibody. Similar results were obtained with guinea-pig and rabbit anti-ox cells sera, which were shown to contain antibodies for ox cells of different specificity to that of the Paul-Bunnell antibody.

The fact that more than one antigen site on the ox cells was involved distinguishes this phenomenon from that observed with human red cells carrying the D^U Rh antigen (Stratton, 1946 ; Stratton and Renton, 1948 ; Race, Sanger and Lawler, 1948). Although D^U cells vary considerably in the strength of their reaction with certain anti-D agglutinating sera, only one antigen site appears to be involved, since those D^U cells which are inagglutinable to anti-D agglutinating sera can nevertheless be agglutinated to a normal degree by other Rh antisera such as anti-C and anti-E. Again, the fact that D^U cells sensitized with " incomplete " anti-D antibodies can be agglutinated by the anti-globulin sensitization technique provides further evidence of the difference between the two phenomena.

The high degree of susceptibility of the sensitized " inagglutinable " ox cells to the clumping action of trypsin emphasizes the fundamental difference in principle underlying the action of trypsin and anti-globulin sera on red cells sensitized with antibodies. This ability of the trypsin technique to demonstrate the sensitization of the " inagglutinable " class of ox cells commends it as a method to be considered for the detection of antibody sensitization in other systems.

Many of these observations have an immediate bearing on the use of haemagglutination in the study of bovine blood groups. They should also be borne in mind in the interpretation of agglutination tests with red cells of other animal species, including man.

Further work on these lines and on the evaluation of the trypsin and anti-globulin techniques in the detection of antibody sensitization is in progress.

SUMMARY.

A property of the red cells of certain oxen has been observed which renders the sensitization of these cells by infectious mononucleosis sera and guinea-pig and rabbit anti-ox red cell sera undetectable by the anti-globulin sensitization test, although they are fully susceptible to lysis in the presence of guinea-pig complement.

The sensitization of this " inagglutinable " class of ox red cell can be detected by treatment with trypsin.

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A COMPARISON OF THE CONGLUTINATING COMPLEMENT ABSORPTION TEST AND THE HAEMOLYTIC COMPLEMENT FIXATION TEST IN THE SEROLOGICAL DIAGNOSIS OF INFLUENZA.

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THE principles of conglutination were originally described by Bordet and Gay in 1906 and a complement absorption test, using conglutinating complement, was devised by Streng (1910). It was not until the reinvestigation of the whole phenomenon by Hole and Coombs (1947*a*, 1947*b*, 1947*c*), however, that an accurate comparison of the absorption of different complements was made. In complement absorption tests with human anti-mallein and anti-typhoid sera they obtained far higher antibody titres with horse conglutinating complement than with guinea-pig haemolytic complement.

Because of the importance of complement fixation tests in the study of virus diseases, Stoker, Coombs and Bedson (1950) have investigated the application of the conglutinating complement absorption test in the diagnosis of lymphogranuloma inguinale, vaccinia and influenza. In this work the most encouraging results were obtained with the influenza virus and its antibodies. Influenza virus antigen, unlike that of lymphogranuloma and vaccinia viruses, was not anticomplementary to horse conglutinating complement, and human antibody titres obtained with it in the conglutinating complement absorption test (C.C.A.T.) were much higher than those obtained in the orthodox haemolytic test (H.C.F.T.). Antisera from only three cases of influenza were examined, however, and a comparison by the two tests of the rise in titre during the illness was possible in only one case. High absolute titres are of little or no diagnostic value if the rise during the course of infection is small.

The purpose of the investigation described in this paper was to compare the sensitivity of the C.C.A.T. and H.C.F.T. with both acute and convalescent phase sera from patients with influenza. Tests were only done with the soluble antigen of influenza viruses A and B, and antibody titres to the virus particle were not investigated.

MATERIALS AND METHODS.

Antigens.

In the preliminary investigation, Stoker *et al.* (1950) used a chloroform extracted mouse lung antigen. In this investigation both influenza A and B soluble antigens were prepared from the chorioallantoic membrane of the chick embryo.

The PR8 strain of influenza virus A and the Lee strain of virus B were used to prepare the soluble antigens. Of a 10^{-4} dilution of infected allantoic fluid 0·1 ml. was inoculated into the allantoic cavity of twelve-day-old chick embryos. These embryos were then incubated at 36° C. for eighteen hours and some allantoic fluid harvested without previously chilling the eggs. This allantoic fluid was then passed undiluted in 0·1 ml. amounts to the allantoic sac of thirteen-day-old embryos which were then incubated at 36° C. for eighteen hours. At the end of this time the embryo and yolk sac were removed and the chorioallantoic membrane harvested. The membranes were washed in three changes of 0·08 per cent azide-saline to remove blood, spilt yolk, etc., drained and weighed. They were then frozen and thawed three times by alternate immersion in an alcohol-solid CO_2 mixture and a 37° C. water bath. Enough 0·08 per cent azide-saline was added to make an approximate 20 per cent W/V suspension of membrane and saline and the mixture was then homogenized in a blender. The suspension was spun lightly and the supernatant removed. The supernatant was adsorbed with washed human red blood cells in a final concentration of 4 per cent V/V cells. The mixture was kept at 2° C. overnight and centrifuged while still cold to remove the red cells and the virus adsorbed onto them. The supernatant was treated with chloroform, 1 drop per ml. (2 per cent), and left overnight at 2° C. to complete the reaction. The resulting precipitate was removed by horizontal centrifugation at 6000 r.p.m. for 30 minutes. This process resulted in a slightly opalescent fluid, which was dried from the frozen state. Tests of a number of batches of such " soluble antigen " have shown that they do not agglutinate red cells and that their complement fixing property is not materially altered by centrifugation for 1 hour in an angle centrifuge at 10,000 r.p.m.

Antisera.

Standard antisera for the titration of soluble antigen consisted of pools of positive human sera from cases of influenza virus A or B infection.

Complement fixation tests.

Guinea-pig and horse serum were used as sources of haemolytic and conglutinating complement respectively. These were stored in approximately 1 ml. quantities at — 20° C. without preservatives.

The techniques for performing the H.C.F.T. and C.C.A.T. were those described by Hole and Coombs (1947*b*) with the modifications introduced by Stoker *et al.* (1950). In the latter investigation, overnight fixation at 2° C. was found to give higher antiserum titres in both tests than fixation for 2 hours at room temperature. The former method was therefore used in all tests, including the preliminary complement titrations.

Titration of complement and selection of sera.

The optimal dilution of the soluble antigens was determined by titration in chess board fashion by both the C.C.A.T. and H.C.F.T., using standard homologous human antisera and two doses of horse or guinea-pig complement. Previous titration of the complements with each antigen dilution showed that neither influenza virus A nor B antigen was anti- nor procomplementary for horse or guinea-pig complement. The results for the influenza virus A antigen are given in Fig. 1. A dilution of 1 : 20 was taken as optimal for the C.C.A.T. and 1 : 10 for the H.C.F.T. These were also the optimal dilutions for influenza B antigen in the two tests.

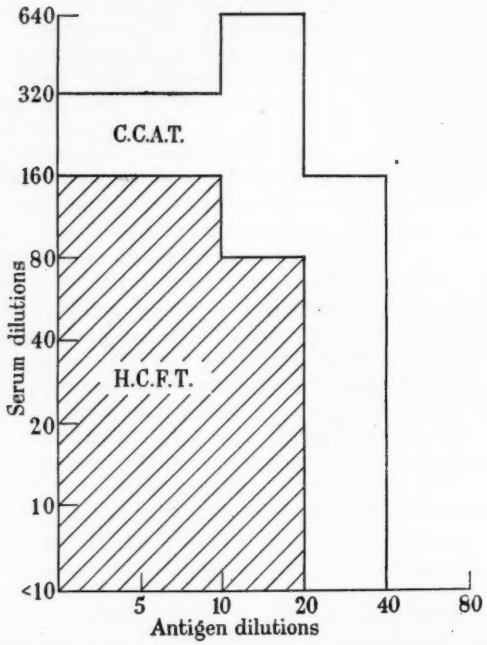

FIG. 1.—Titration of influenza A convalescent serum by the haemolytic complement fixation test (H.C.F.T.) and conglutinating complement absorption test (C.C.A.T.) with varying dilutions of influenza A antigen.

Twelve pairs of acute and convalescent influenza A antisera were then titrated with influenza A and B antigens by the H.C.F.T. and C.C.A.T. On the basis of previous routine H.C.F.T.s the diagnosis of influenza A was considered definite in eight of these cases. The remaining four, the "doubtful" group, were from patients with "clinical influenza." Four of the patients in the "positive" group and two patients in the "doubtful" group were from an institutional outbreak in which influenza virus A had been isolated. Six single specimens of serum from apparently healthy students were also tested in the same way. All comparative tests on pairs of sera were put up on the same day.

RESULTS.

The results of these tests are shown in Table I. In general, there was a good correlation between the H.C.F.T. and C.C.A.T. both in absolute titres and in detectable rise of antibody. Out of the 30 specimens of serum examined, 13 were anticomplementary at dilutions of 1 : 5 or higher. Of these, 3 sera affected

TABLE I.—*Comparison of Haemolytic Complement Fixation Test (H.C.F.T.) and Conglutinating Complement Absorption Test (C.C.A.T.) in Influenza.*

		Titres of acute and convalescent human sera.							
		H.C.F.T.				C.C.A.T.			
	Serum.	A. antigen.	B. antigen.	Anti-comp. activity.†	Detectable rise in A antibody.	A. antigen.	B. antigen.	Anti-comp. activity	Detectable rise in A antibody.
Influenza	Br acute	0	0	0	× 64	0	0	0	× 128
	Br conval.	160	0	0		320	5	5	
	Ty acute	5	0	0	×32	10	10	10	× 64
	Ty conval.	160	20	10		640	20	20	
	La acute	0	0	0	× 16	40	20	0	× 8
	La conval.	40	0	0		320	20	20	
	Me acute	20	20	0	× 4	40	40	20	× 16
	Me conval.	80	20	0		640	40	40	
	St acute	10	10	10	× 8	0	0	0	× 128
	St conval.	80	10	0		320(Z).	0	0	
	Se acute	10	10	10	× 4	40	20	40	× 4
	Se conval.	40	5	0		160	20	10	
	Tn acute	5	0	0	× 8	0	0	0	× 16
	Tn conval.	40	0	0		40(Z).	0	0	
	Le acute	0	5	0	× 16	0	0	0	× 16
	Le conval.	40	10	5		40	0	0	
Doubtful Influenza	Fe acute	0	5	0	0 (anti-comp.)	0	0	0	0
	Fe conval.	20	10	10		0	0	0	
	Re acute	10	10	5	0 (× 4)	0	0	0	× 32
	Re conval.	0*	0	0		80(Z).	0	0	
	Be acute	20	40	0	× 2	20	40	0	×
	Be conval.	40	40	0		40	80	0	
	Ca acute	20	10	10	0	40	40	40	0
	Ca conval.	10	0	0		80	0	0	
Normal adults	No. 2	0	5	0	..	0	10	0	..
	„ 3	0	0	0	..	0	0	0	..
	„ 6	0	0	0	..	0	10	0	..
	„ 9	10	0	0	..	10	0	0	..
	„ 11	0	0	0	..	0	0	0	..
	„ 12	20	5	0	..	40	10	0	..

Antigens A and B used at optimum = 1 : 20 for C.C.A.T. ; 1 : 10 for H.C.F.T.
0 = <5 ; Z = prozone of inhibition of fixation ; * = 1 : 40 with antigen at 1 : 5 ; † = anticomplementary activity.

both complements, 4 affected guinea-pig complement alone, and 6 horse complement alone. The titre of anticomplementary activity was never more than 1 : 10 for guinea-pig complement, but was 1 : 40 in 3 instances for horse complement. None of the sera from students were anticomplementary, but these had been freshly drawn, while sera from influenzal patients had been stored at — 10° C. for a year. The absence of parallel and marked rises in titre to influenza virus B soluble antigen excludes the possibility that the rises in titre were due to a non-specific anticomplementary summation effect with the sera in the presence of chorioallantoic membrane. Both the antigens had been prepared in the same way and presumably contained similar amounts of egg protein.

In the group of 8 definite cases of influenza, the antibody titre in the convalescent specimens from Tn and Le was the same by both tests, but in the remainder

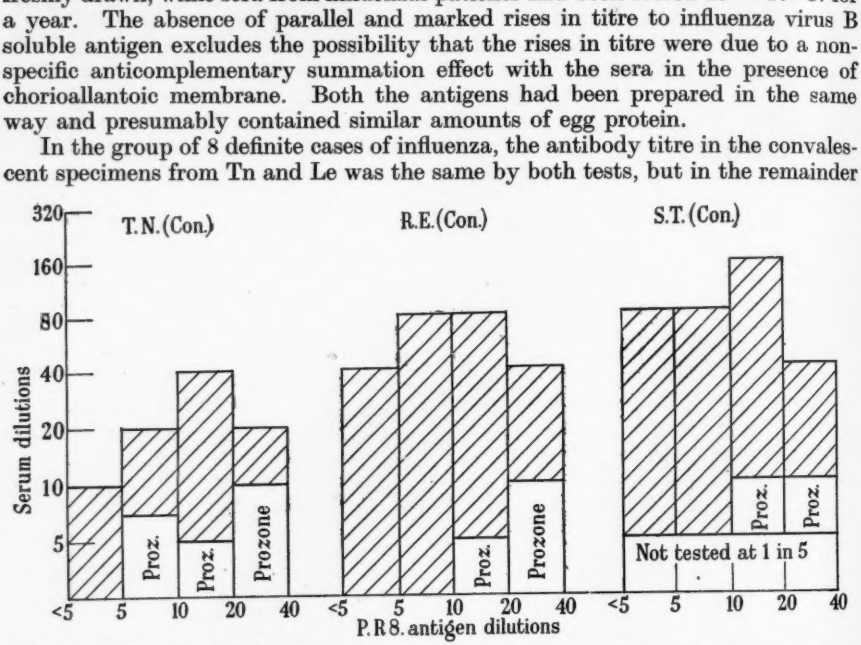

FIG. 2.—Effect of varying dilutions of influenza A antigen on prozone of inhibition of fixation of conglutinating complement obtained in titration of convalescent sera, T.N., R.E. and S.T.

the C.C.A.T. was 2 to 8 times more sensitive. The detectable rise in titre of antibody was the same by both tests in the pairs of sera from Se and Le. In the case of La the H.C.F.T. gave twice the rise shown by the C.C.A.T., but in the remaining five cases the rise in titre shown by the C.C.A.T. was 2 to 16 times that shown by the H.C.F.T.

In 2 of the 4 doubtful cases, neither the H.C.F.T. nor the C.C.A.T. showed any rise of titre. A two-fold rise was detected by both tests in another doubtful case, Be, but this is hardly significant, particularly in view of the cross-reaction with influenza virus B antigen. The remaining doubtful case, Re, is of some interest because the H.C.F.T. was apparently negative, while a 32-fold rise (from less than 1 : 5 to 1 : 80) was shown by the C.C.A.T. When retested, however, with a 1 : 5 dilution of influenza virus A antigen instead of 1 : 10, the titre in the H.C.F.T. was 1 : 40. The optimal antigen dilution was rechecked with two other antisera and still found to be 1 : 10, as determined originally, but apparently for this particular serum (Re) the optimum was 1 : 5.

Prozones of conglutination in low serum dilutions were present in three convalescent sera (St, Tn and Re) tested by the C.C.A.T. It was thought that these

might be due to a factor in some normal human sera which masks absorption of conglutinating complement (Blomfield, Coombs and Hole, 1950). A chess board titration of each serum, however, showed that the zone disappeared as the concentration of antigen increased (Fig. 2). This suggests that the effect might have been due to inhibition of fixation in the region of antibody excess. Nevertheless, the presence of this zone did not depend on a high final antibody titre, in fact two sera with the effect had rather low titres of 1 : 40 and 1 : 80 respectively, whereas two with titres of 1 : 640 showed no prozone.

Cross-fixation with influenza virus B antigen was detected in some sera by both tests, but in no instance was there a significant rise in titre during illness. Of the 6 apparently normal individuals, 4 had serological evidence of past exposure to influenza virus A or B. In one of these (No. 6) the influenza B antibodies were detected by the C.C.A.T. only, but the remainder were revealed by both tests without significant difference.

It would appear from these results that the sensitivity of the C.C.A.T. is generally greater than that of the H.C.F.T. in influenza virus A infection, as judged by the rise in antibody titre during infection and final titre in convalescence. The difference was rarely greater than four-fold, however, especially when comparing rise of titre. In one doubtful case the C.C.A.T. was positive and the H.C.F.T. apparently negative, but by varying the antigen concentration antibodies were revealed in the H.C.F.T. as well. At present, therefore, it seems unlikely that fixation of conglutinating complement will replace the orthodox haemolytic complement fixation test in the routine serological diagnosis of influenza. Nevertheless, the greater sensitivity of the test might assist in the elucidation of particular problems where other tests have failed.

SUMMARY.

The conglutinating complement absorption test and haemolytic complement fixation test were compared for detection of human influenza virus A antibodies.

Acute and convalescent sera from eight definite and four doubtful cases of influenza A infection, and single specimens from six normal individuals were examined by both tests with influenza virus A and B soluble antigens.

The results showed that the conglutinating complement absorption test was usually the more sensitive both in the detection of antibody rise during infection and of final titres in convalescence.

This increased sensitivity, however, was variable and in the present state of knowledge was not sufficiently great to justify replacement of the orthodox haemolytic complement fixation test by the conglutinating complement absorption test in routine laboratory diagnosis.

Thanks are particularly due to Dr. R. R. A. Coombs for his helpful criticism and advice and for the supply of many of the materials used in the tests.

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THE CALCIUM REQUIREMENT OF A TYPHOID BACTERIOPHAGE.

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IN the course of work with a strain of *Bact. typhosum* and its phage it was found that normal lysis and free phage production took place in peptone media, but that the results on a simple ammonia medium were entirely unpredictable. Lysis detectable by the eye or even by a Hilger "Spekker" absorptiometer seldom occurred and production of free phage was usually poor. This was all the more curious because the phage plated on the synthetic medium *plus* agar produced plaques almost as well as on peptone agar.

If in the course of growth in the ammonia medium traces of peptone were added, visible lysis set in rapidly and therefore peptone was fractionated to find the active factor in it. The first step was a 66 per cent alcohol precipitation. One fraction contained inorganic material which was active in traces. This contained Ca, Fe and Cu among other elements and, these being tested, Ca was found to be the factor responsible. Calcium chloride was then used in the ammonia cultures and always produced lysis within an hour or two of its addition at any point in the log phase of growth.

This paper is a study of this matter.

PREVIOUS WORK.

Calcium has been implicated, directly or indirectly, as a factor in phage growth for a number of years.

It was found by Stassano and de Beaufort (1925) that citrate inhibited phage growth. This citrate effect was confirmed by Andrewes and Elford (1932). In neither of these papers, however, were experiments carried out directly on the effect of calcium.

Bordet and Renaux (1928), working with Shiga phage, found that if broth was "highly alkalinized" in order to remove the phosphate precipitate and then used for the growth of phage, no lysis took place. But lysis followed the addition of one drop of 1 per cent $CaCl_2$ to 5 ml. culture if this was added before the culture had progressed too far. Further, normal broth could be made deficient for phage growth by adding 1 : 1000 oxalate. Other phages tested were not inhibited by oxalate, but, in view of the fact that oxalate does not remove all calcium from solution, they thought it possible that all phages might require calcium in varying concentrations.

Wahl (1946) and Wahl and Blum-Emerique (1949) have described two phages which required calcium, but this was only effective in a narrow zone of concentrations.

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Delbruck (1948) found calcium to be a necessary component in the adequate adsorption of phage T_4 on Coli B, in the sense that the particles adsorbed were increased in number five times by addition of 4 μg./ml. $CaCl_2$.

Puck (1949) has shown that a Coli phage can be " sensitized " by dilution in distilled water or dilute buffer, but not if calcium is present. These sensitized phages are inactivated when adsorbed by bacteria, and form no plaques when plated.

Adams working with the T5 phage and Coli B also found that lysis and phage production did not occur in an ammonia medium in the absence of added calcium.

TECHNICAL DETAILS.

Host bacterium.—All work has been carried out on a strain of *Bact. typhosum* selected from No. 3390 N.C.T.C.

Typhoid phage.—This was derived from a phage obtained from Brigadier J. S. K. Boyd (Wellcome Laboratories) active on No. 3390 N.C.T.C. The actual phage preparation used in this work were not lysates in the ordinary sense, because lysis did not take place. They were supernatants of cultures of bacteria and phage made in the culture medium described below. They were heated at 58° for forty minutes to destroy phage-resistant strains.

The initial material was obtained by plaque selection from platings on single-colony isolations of the bacterium. The plaques produced are small.

Culture medium.—All work has been carried out on the following medium.

KH_2PO_4	9·0 g.
Ammonium chloride	·5 g.
Ammonium sulphate	·5 g.
Anhydrous magnesium sulphate	·01 g.
N/1 NaOH	ca. 63 ml.
Double distilled water	to 500 ml.

This was autoclaved in 100 c.c. lots at pH 7·6 in eight-ounce " medical flat " bottles. For use this mixture was diluted with water 1/2 and 8 ml. 11·25 per cent glucose (autoclaved in water) added per 200 ml.

A solid medium was made by diluting with 3 per cent Difco agar in place of water.

Culture technique.—Erlenmeyer flasks were used for large quantities, the amount of culture being 1/10 of the flask volume. The flasks were shaken in the hot room.

For smaller, 10 to 15 ml., experimental volumes the T-shaped test tube of Monod was used. This was rocked mechanically in a water bath.

The vessel used by Dr. J. Monod (Institut Pasteur) is an inverted T-shaped apparatus with the opening at the top and the culture in the horizontal arms to facilitate aeration when the tube is rocked in the water bath. It is square in section and optically worked, so that the opacity of the culture can be measured directly in the absorptiometer. Dr. W. E. van Heyningen (School of Pathology, Oxford) has modified the vessel so that it is made of Pyrex glass, circular in section, and this modification has been used in the present work. We are indebted to Dr. Monod and Dr. van Heyningen for this unpublished information.

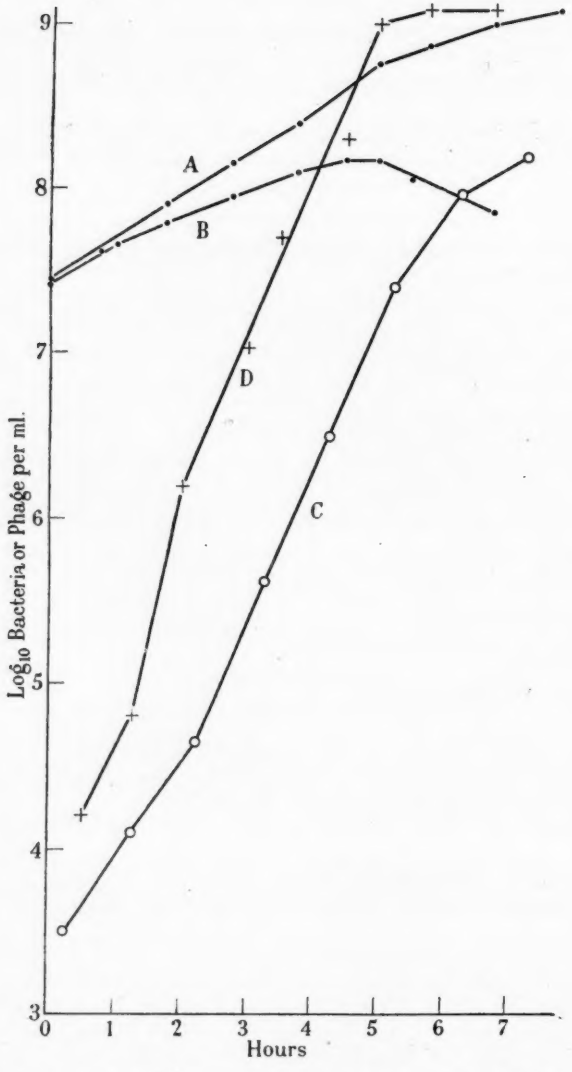

FIG. 1.—Effect of calcium on lysis and phage production. A = Bacterial growth without added calcium, but with added phage; C = phage production in the same whole culture; B = bacterial growth with added calcium (M/3500) and phage showing lysis; D = phage production in the same whole culture.

Estimations of bacteria.—All bacterial estimations were made by opacity, using Hilger's " Spekker " absorptiometer, so modified that the Monod's tubes could be used in place of cells. The reading from the calibrated tube could then be referred to bacteria per ml. by means of standard curves. In an uncomplicated growth experiment this method gave results which closely agreed with bacterial counts, but, of course, during phage lysis, for instance, it did not follow that the opacity reading corresponded to viable bacteria per ml.

Estimation of phage.—Dilutions of phage were always made in the culture medium. Aliquots were plated by the two-layer method, using an agar (2 per cent) containing 1 per cent Evans' peptone and M/40 glucose for the bottom layer. The top layer was prepared by mixing 0·5 c.c. phage dilution with 0·5 c.c. bacterial suspension (ca. 5×10^8/ml.) and after one minute adding 2 c.c. 1·5 per cent glucose-peptone agar. The whole was then poured onto the bottom layer.

The dilutions of phage, made in the culture medium, were arranged to give about five hundred plaques per plate and two plates were used for each dilution.

EXPERIMENTAL.

Effect of Calcium on Lysis and Phage Production.

Fig. 1 shows (Curve A) that the generation time of the bacterium in the medium containing phage but no added calcium was 72 minutes, which does not differ from a culture without phage. The growth rate of the culture (Curve B) with both phage and calcium added soon became reduced and at 4½ hours lysis became visible. The production of phage in the whole culture was more rapid with calcium and a higher yield was obtained. At 5 to 6 hours phage production fell off in the culture without calcium because the log phase of bacterial growth had come to an end.

It is clear that visible lysis can only take place when most of the bacteria in a culture are infected, i.e. when the phage titre approaches or exceeds the bacterial count. When calcium is not present the phage titre (Curve C) has only attained about 1/10 of the bacterial count (Curve A) before the culture ceases, and this is sufficient explanation for the absence of visible lysis.

The Effect of Substances which React with Calcium.

Fig. 1 indicates that calcium added to a culture has a marked stimulatory action on lysis and phage production and suggests the possibility that phage produced in the absence of added calcium is due to calcium as an impurity in the medium. Experiments were therefore carried out with substances which might be expected to reduce the number of available calcium ions.

Phosphate.

Wahl and Blum-Emerique (1949) have shown that a certain Coli phage, S13, would not multiply on their ordinary synthetic medium, which contained M/10 buffer even when calcium was present, but they found multiplication when the buffer concentration was greatly reduced. For instance, in the presence of a "très faible" concentration of $CaCl_2$ and M/215 buffer (0·442 g. PO_4/litre) the initial titre of phage was 1×10^5 and the final titre $3{\cdot}5 \times 10^5$/ml. With M/270 buffer, the final titre was 2×10^7; with M/540, $1{\cdot}5 \times 10^8$, and with M/1610, $4{\cdot}5 \times 10^8$.

We have tested the effect of phosphate concentration in the medium without and with added calcium. It was not practicable to reduce the phosphate greatly because then complication arose from changes in pH. Our ordinary medium contained M/15 phosphate and this was tested against the same medium containing, however, M/30 phosphate. In both media the bacteria grew at the same rate. When the cultures had reached 1×10^8/ml., phage was added to an aliquot of each to make 1×10^5 phage particles/ml. As usual no lysis was observed in the M/15 buffer tube during the period of growth, but in the M/30 buffer tube

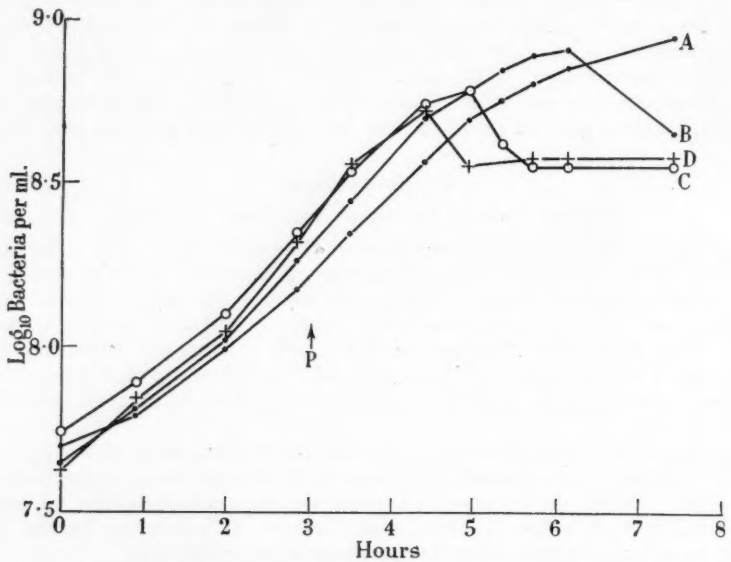

FIG. 2.—The effect of phosphate concentration on lysis. A = M/10 phosphate; B = M/15 phosphate; C = M/30 phosphate; D = M/75 phosphate. Phage added at P.

lysis started three hours after addition of phage. At 4½ hours after addition of phage, the partially lysed tube, when plated, gave $1{\cdot}1 \times 10^9$ plaques/ml. in the whole culture and $7{\cdot}6 \times 10^8$ in the supernatant. On the other hand, the M/15 buffer tube gave at the same time $5{\cdot}8 \times 10^8$/ml. in the whole culture and $2{\cdot}9 \times 10^8$ in the supernatant. Thus reduction in phosphate caused a two-fold increase in phage production and a larger proportion free in the culture.

The effect of phosphate was also tested quantitatively in the presence of added calcium (M/4000).

Fig. 2 shows that lysis did not occur with M/10 phosphate (Curve A), even though calcium was added but that on reduction of phosphate lysis set in progressively sooner.

The results of these experiments accord with the view that available calcium ions can be reduced by increasing the phosphate concentration and so reducing phage production and lysis.

Oxalate.

The action of oxalate on phage production was studied in two ways.

(*a*) The effect on lysis induced by added calcium.

(*b*) The effect on phage production in the absence of added calcium.

As a preliminary it was found that concentrations of oxalate as high as M/100 had no effect on the bacterial growth curve, except in so far as the higher concentrations caused precipitates in the presence of added calcium. These caused the opacity curve to stand at a higher level, but did not interfere with the slope of the curve.

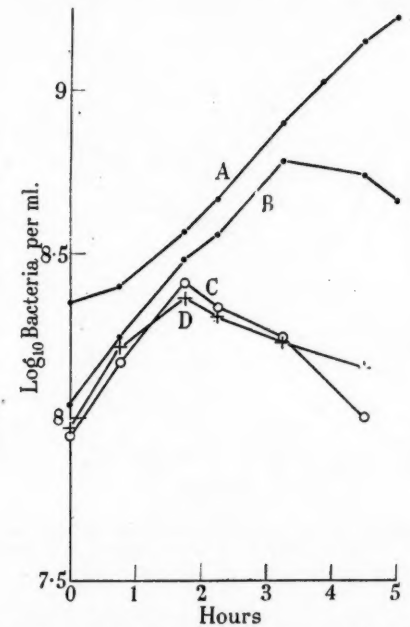

FIG. 3.—The effect of oxalate on phage lysis induced by calcium (M/3500). A = Bacterial growth (plus oxalate precipitate) with M/100 oxalate; B = bacterial growth with M/200 oxalate; C = bacterial growth with M/400 oxalate; D = bacterial growth control without oxalate.

Four tubes were set up (Fig. 3) containing 10^8 bacteria, 2×10^4 phage/ml., and M/3500 calcium. One tube (D) contained no oxalate and the others, three concentrations of oxalate. It is seen that M/400 oxalate had little effect, but that M/200 caused a marked delay in lysis, while M/100 did not lyse. The effect of oxalate is therefore to delay the lysis induced by calcium, even though no precipitate is formed. In the high concentrations visible lysis is abolished.

The effect of oxalate on phage production was shown by diluting phage in the medium containing no added calcium, but different concentrations of oxalate, namely, M/420, M/240 and M/120. A control was set up without oxalate. The cultures were then incubated, the bacterial growth recorded and samples plated

for phage concentration from time to time. The bacterial growth curves with or without oxalate were practically identical, starting at 1×10^8. The initial phage concentration was approximately 1×10^4. Without oxalate there was the normal continual increase similar to that shown in Fig. 1c. With M/240 oxalate there was a marked delay before increase started ; with M/420 there was even a slight fall in the phage content followed by an increase. The rate of increase did not much differ from that with M/420, nor even from the control. With, however, M/120 oxalate there was a heavy fall in concentration of phage. This also was followed by an increase, but this was much slower.

Fig. 4 shows the effect of another experiment with M/100 oxalate in detail. The initial titre of phage was $1{\cdot}5 \times 10^4$/ml. In two hours this fell to 1/10 and

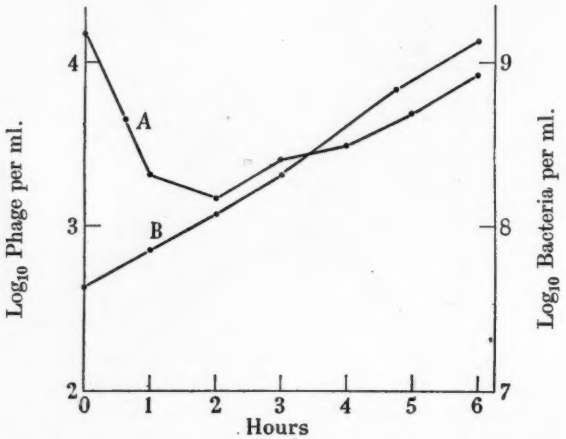

FIG. 4.—The effect of oxalate (M/100) on phage production without added calcium. At the start there were $4{\cdot}3 \times 10^7$ bacteria and $1{\cdot}5 \times 10^4$ phage. A = Phage production ; B = bacterial growth.

then an increase set in at a rate which is some five times slower than without oxalate.

These experiments show that phage is still produced in the presence of oxalate, though more slowly especially in the higher concentrations. Phage lysis induced by calcium is also delayed by higher concentrations of oxalate and eventually abolished. Addition of oxalate thus produces the same effect as reduction in calcium, namely, a fall in phage production and abolition of lysis.

Attention is called to the initial fall in phage titre shown in Fig. 4. It is clear that in the presence of bacteria and M/100 oxalate 90 per cent of the phage particles are rapidly inactivated. This matter will be referred to later.

THE PRESENCE OF CALCIUM IN CULTURE MEDIA.

The experiments described suggest that phage production by the system in use is absolutely dependent upon the presence of free calcium ions. Failure to

show a conclusive result seemed likely to be due to undisclosed calcium ions in the culture medium.

The following experiments were therefore carried out:

The medium freshly prepared from "Analytical Reagent" chemicals and double distilled water did not give a reaction for calcium by the oxalate test either at its normal strength or after a ten-fold concentration by evaporation. After autoclaving in eight-ounce "medical flat" bottles no reaction was given, but a ten-fold concentration showed calcium to be present in some samples. In view of this, a bottle of medium was autoclaved for twelve hours at 15 lb./sq. in.

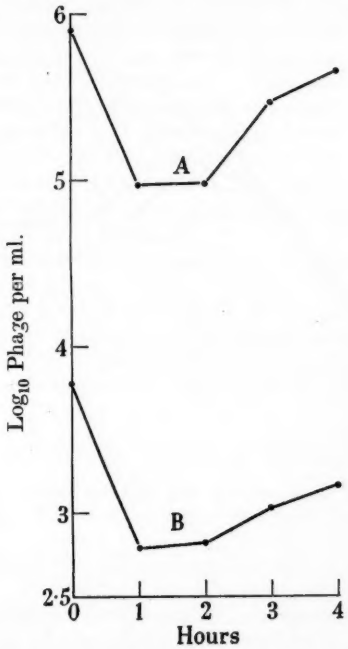

FIG. 5.—Production of phage in "calcium-free" medium. A = Phage production from an initial concentration of $7{\cdot}9 \times 10^4$ phage/ml.; B = phage production from an initial concentration of $5{\cdot}8 \times 10^3$ phage/ml.

and tested after concentration. A strong positive reaction was given. Calcium was also found in the cotton-wool used to plug the bottles. It appeared that calcium could be introduced into the medium from cotton-wool, fibres of which fall into the medium, and from the bottles during autoclaving. The medium was therefore prepared and sterilized in "Pyrex" flasks fitted with beakers instead of plugs. Further, the N/1 soda used for neutralizing was kept in paraffined bottles. After 24 hours' autoclaving no calcium was detectable in the medium thus prepared.

The method used by Bordet and Renaux (1928) to decalcify a culture was also tested.

A batch of the ammonia medium made in soft glass was divided into two. One part was kept as a normal control and the other was adjusted red to phenolphthalein and warmed to about 90°. A small precipitate was filtered off, the pH readjusted to 7·6 and the medium finished. These two batches of medium were then used to test inactivation of phage. Phage diluted in each medium

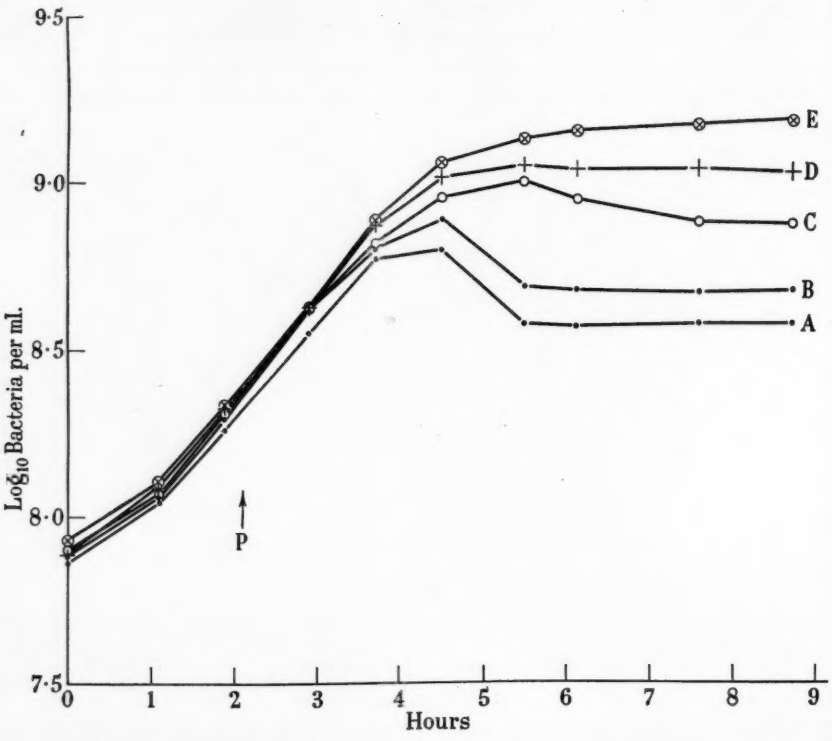

FIG. 6.—The effect of calcium concentration on lysis. A = Calcium M/2100; B = calcium M/4200; C = calcium M/10,500; D = calcium M/21,000; E = no calcium. Phage added at point P.

was mixed with bacteria grown in each medium and after one and two hours plated for active phage in the whole culture.

In the medium made with soft glass, the plaques rose as usual from $1{\cdot}7 \times 10^6$ to $3{\cdot}3 \times 10^6$ in one hour, and to $1{\cdot}7 \times 10^7$ in two hours. In the decalcified medium the plaques fell from $1{\cdot}6 \times 10^6$ to $4{\cdot}7 \times 10^5$ in one hour and then rose to $4{\cdot}6 \times 10^6$.

Thus a process which is known to remove calcium, transforms an adequate medium to the same condition as one which contains oxalate.

Experiments on "Calcium-free" Media.

Experiments were now conducted in which all glassware used was "Pyrex," and cotton-wool was eliminated as far as possible. Otherwise the materials and conditions were precisely the same.

This alteration had no effect on the rate of growth of the bacterium in use.

Fig. 5 shows the rate of phage production in the whole culture under these conditions. It is seen that the curves resemble closely that obtained with oxalate in the normal medium (Fig. 4). There was an initial heavy fall in phage content, and a slow and small increase in phage later. This small increase may be compared with the increase shown in Fig. 1, Curve C. The only difference between the experiments was the avoidance of known sources of calcium in Fig. 5.

Nevertheless, the fact that phage growth took place at all indicates, in our view, that calcium ions were still present. This, however, is inevitable under present conditions because our phage itself, although produced without added calcium, was made in part in soft glassware and can only be produced in this way, or by adding calcium. In fact the curves in Fig. 5 indicate that the concentration of phage lysate affects the result. In Curve A the rate of phage growth is faster and the number of calcium ions added with the phage is one hundred and thirty-six times greater than in Curve B.

The concentration of calcium necessary to produce lysis was tested in the medium prepared in hard glass. Fig. 6 indicates that the effect of calcium is quantitative. It is most marked at M/2100, which is the highest concentration which can be used without producing a precipitate, and it is least marked in the lowest concentration. There is no evidence of an optimum concentration, above or below which the effect is less, as recorded by Wahl and Blum-Emerique (1949).

Inactivation of Phage by Bacteria in the Absence of Calcium.

Attention has been called to the inactivation of phage by bacteria in the presence of oxalate (Fig. 4) after removal of calcium phosphate or in the absence of calcium (Fig. 5).

Puck (1949) has shown that T 1 phage diluted with distilled water or with dilute buffer becomes "sensitized" in the sense that it is inactivated when brought into contact with *E. coli* B and forms no plaques when plated. This sensitization is prevented by the presence in the diluting fluid of $CaCl_2$ M/5000 or M/10,000, but not by M/100,000. The phenomenon noted by us, thus resembles closely that found by Puck.

Our experiments suggest that if, under the conditions of the test, phage is exposed to bacteria in the absence of calcium it is permanently inactivated, while if it is exposed in the presence of calcium it is not. This being so, it is clear that the phage associated with bacteria in a "calcium-free" medium and relying only on traces of calcium carried over with the lysate, cannot multiply effectively. A large fraction of each burst will be inactivated in the absence of adequate calcium and the overall increase of active phage will be little or none.

CONCLUSIONS.

A particular typhoid phage is permanently inactivated on exposure to bacteria in the absence of calcium.

Thus it requires calcium for continued propagation under the conditions imposed.

Practically all the phage estimations in this work have been carried out by Miss Audrey Hearle. We have pleasure in thanking her for this and other assistance.

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THE ALIMENTARY TRACT OF RATS WITH INTESTINAL CULS-DE-SAC.

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BARKER AND HUMMEL (1939) reviewed the findings in fifty-one cases of Addisonian anaemia associated with intestinal strictures and anastomoses. This group comprised a variety of lesions. The anastomoses were chiefly entero-enterostomies and entero-colostomies although occasional gastro-jejuno-colic and gastro-colic fistulae were reported. The strictures were single or multiple, and more than a third of them were of tuberculous origin. Watson, Cameron and Witts (1948) and Cameron, Watson and Witts (1949) were able to produce a similar condition in experimental animals. A macrocytic anaemia developed in adult albino rats in which they had made a blind loop or cul-de-sac in the small intestine. The anaemia was characterized by a high mean cell diameter and an increased variability in the cell diameter. The white blood cells were normal. The anaemia developed at different periods after the operation, according to the operative technique, but once it appeared it rapidly progressed. The anaemia was relieved by treatment with pteroyl-glutamic acid, although the untreated condition was usually fatal and the effects of liver extract (Anahaemin) and vitamin B_{12} were equivocal (Cameron, Callender, Watson and Witts, 1949).

In early experiments (Group A) the blind loop was so arranged that peristalsis tended to empty it, while in later experiments (Group B) the peristaltic arrangement was such that the blind loop tended to be self-filling (Fig. 1). The proportion of animals developing macrocytic anaemia was greater in the latter group. We have studied the pathology of the gastro-intestinal tract of some of these animals to find out whether the mechanical lesions were associated with any gross or histological changes, which might elucidate the nature of the anaemia and explain why it developed in only a small proportion of the earlier group. In all the experiments described in this paper the blind loop corresponded approximately with the middle third of the small intestine and was about 12 inches in length. In Group B, where the by-passed intestine inevitably becomes greatly distended, a blind loop of this size imposes a heavy strain on the rat. In subsequent work we found that a self-filling loop of 3 or 4 inches is adequate to produce anaemia and is associated with a much lower mortality; the changes in these shorter loops are similar to those which will be described for Group B.

Gross Pathology.

Group A.—Autopsies were performed on seven of the anaemic animals in this group. The feature in each case was the presence of a dilatation of the

small intestine at the site of the anastomosis. The dilatations varied in size from 1 to 3 cm. in diameter, and extended a short distance up the blind loop. The rest of the loop was collapsed and empty, but otherwise intact. Except for this dilated part the gut appeared quite normal. The stomach also appeared to be normal, and when stomach and intestines were opened no abnormality of the mucosa was discovered. No lesions of the liver, spleen or tongue were seen.

Twelve of the rats in Group A, which had survived the operation for six to twelve months without developing any anaemia, were sacrificed and their alimentary tracts were examined. In none of these cases was there any dilatation

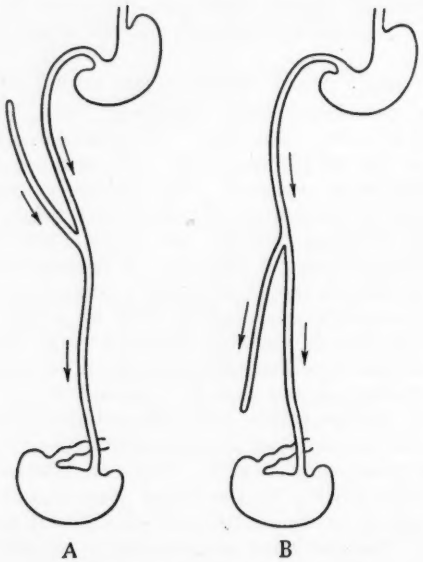

FIG. 1.—Diagram to show direction of peristalsis in Groups A and B.

at the site of anastomosis. The blind loops were patent, but empty and collapsed throughout their lengths. On opening the stomach and gut no mucosal lesions were detected, nor was any abnormality of tongue, liver or spleen observed. These findings suggested that the presence of a dilatation in the small intestine played an essential part in the development of the anaemia, and that a self-emptying blind loop by itself had no such effect.

Group B.—Post-mortem examination of seven anaemic rats in this group revealed in each case a gross dilatation and hypertrophy of the blind loop and an increase in the vascularity of its mesentery. The blind loop varied in diameter from two to three centimetres throughout its length, and was tensely filled with thick dark liquid material. The stomach and the rest of the gut appeared normal. When the stomach and intestines, including the blind loop, were opened no abnormality of the mucosa was evident. The tongue, liver and spleen seemed normal in each case.

Histological Pathology.

The exact time at which an animal died could rarely be determined. Material from such an animal was not satisfactory for histological studies, because autolysis of the mucosa of the alimentary tract commences shortly after death. In order to secure fresh material for the purpose of this investigation, two rats from

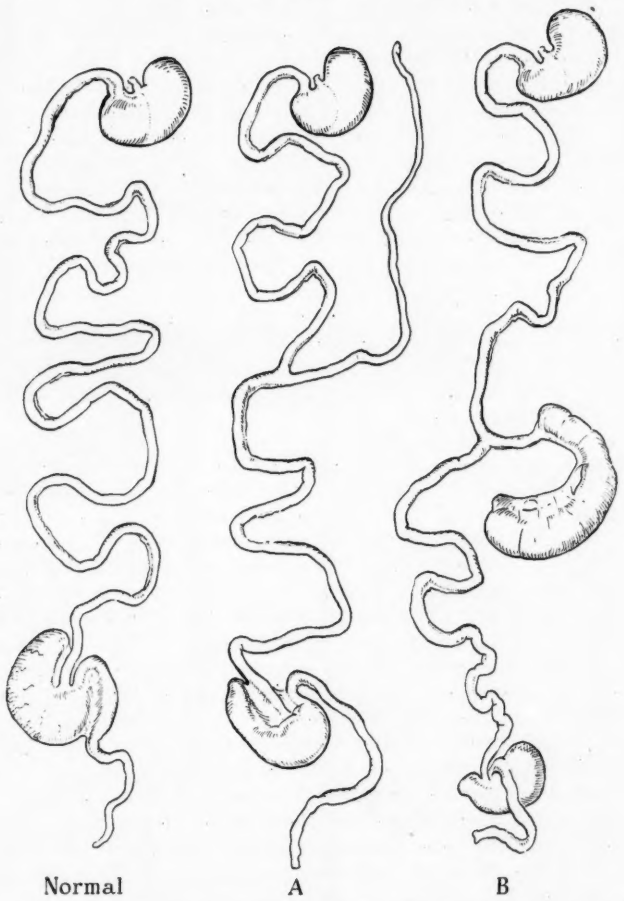

FIG. 2.—Drawing of normal rat intestine, and examples from Groups A and B.

Group A and five from Group B were sacrificed at a time when their blood pictures showed a well-marked macrocytic anaemia. None of these animals was suffering from complicating infections. Seven normal rats, which had been maintained under the same conditions and which were of the same age as the anaemic animals, were sacrificed to serve as controls. Because of the suggestion by Eros (1933) and Jacobson (1939) that the argentaffin cells of the gastro-intestinal tract play

a vital role in the pathogenesis of liver-deficiency macrocytic anaemias in man, it was decided to investigate these cells in the present experimental material, in addition to making the routine histopathological studies.

Under ether anaesthesia the animal's abdomen is widely opened. The rectum is tied off low down and the oesophagus is ligated just below the diaphragm. Ten per cent formal-saline solution is quickly injected through a large-bore needle into the lumen of the gut until the entire segment between the ligatures is filled with the fluid. This segment, comprising the stomach, small intestine and large intestine, is rapidly freed from the abdominal cavity and placed in fixative (10 per cent formol-saline). Specimens of tongue, liver and spleen are taken, and placed in a similar fixative solution. The specimen of stomach and intestines is removed from the fixative, stripped of its attachments and opened throughout its length along its antimesenteric border. It may also be divided into shorter segments for convenience of further preparation and study. Adapting the technique used by Magnus (1937) for preparing human stomach material, each segment is rolled from below upwards to form a " Swiss roll " (Fig. 3). The rolls are replaced in the fixative for forty-eight hours, and then embedded in paraffin. Sections are cut in the usual way and are stained by the following methods : haematoxylin and eosin ; Masson's (1914) silver impregnation method ; Prussian blue reaction for haemosiderin.

Fish, Malone and Richter (1944), described the anatomy of the rat's tongue. Donaldson (1924) noted that tonsils and gall bladder are absent in the rat. The cardiac region of the stomach is poorly defined because of the considerable encroachment of squamous epithelium into the gastric cavity. With these exceptions gross examination of the present material confirmed the general similarity of the rat's alimentary tract to that of man, and histological studies revealed analogous structure. Dawson (1944), using Bodian's (1936, 1937) technique, demonstrated argentaffin cells in the rat's stomach and described the distribution of these cells in the gastric mucosa. Dawson's observations were confirmed in the stomachs of each of the seven normal animals examined. In the intestines the argentaffin cells were less frequent, and were scattered at intervals with no suggestion of a numerical increase towards the lower ileum or colon.

Group A.—Sections of the spleen from the two animals in this group showed haemosiderosis in excess of the normal. The sections of stomach and intestines appeared quite normal, and nowhere was there any evidence of atrophy or inflammation. Argentaffin cells were present in normal numbers and distribution in the stomachs and intestines of both these animals. No changes were seen in the livers or tongues.

Group B.—The sections of spleen from the five animals in this group also showed excess haemosiderosis. Sections of blind loop showed that the increase in thickness noticed in the gross specimens was due to hypertrophy of all the coats (Fig. 4). The villi were thicker and longer and the glands were deeper than normal. The individual cells, however, appeared quite normal and were not increased in size. There was no increase in the relative frequency of mucus-secreting cells. The stomach and the remainder of the small intestine appeared normal. Nowhere was there any evidence of inflammation. Argentaffin cells

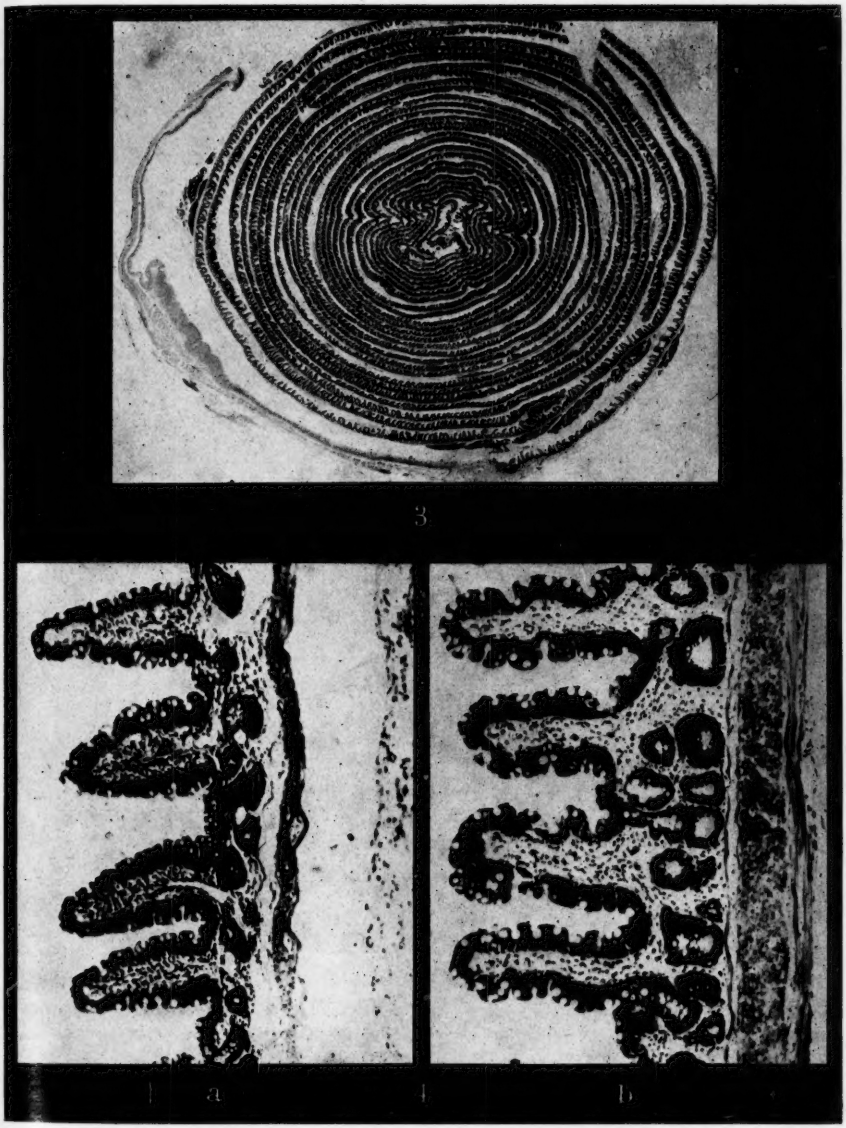

FIG. 3.—The "Swiss roll" technique for sections of the intestine, H. & E. × 3½.

FIG. 4.—a: Section of normal rat ileum. b: Section from a blind loop showing hypertrophy of all coats.

Cameron, Watson and Witts.

were present in normal numbers and distribution in the stomachs and intestines of each of these animals. The livers and tongues appeared to be normal in every case.

DISCUSSION.

When macrocytic anaemia is found in association with intestinal stricture or anastomosis in man, stagnation of the contents of the small intestine, either above a stricture or in a by-passed loop, has been the most constant pathological finding. This phenomenon is reproduced in the experimental animals. The mere exclusion of approximately one-third of the small intestine has no specific effect, and development of the anaemia seems to depend on the accumulation and stagnation of intestinal contents in the loop. Self-filling loops are much more efficient in this respect than self-emptying ones, and when the anaemia occurs in association with the latter it can usually be explained by a constriction which hinders the emptying of the loop.

Rüdel and Tönnis (1932), who made self-filling culs-de-sac of a similar type to ours in dogs, found that the stomach, intestine, and the wall of the cul-de-sac, which was dilated and hypertrophied, showed no inflammatory changes, save in the exceptional case where rupture had occurred. Estes and Holm (1932), also carrying out a similar procedure in dogs, found ulcerative enteritis in both the loop and the neighbouring intestine, but Pearse (1934) found that ulceration and perforation occurred only in those dogs in which a foreign body, held by the debris in the lumen, had produced pressure necrosis; the mucosa of the loop was usually intact, and the stomach and intestine were normal. We have seen perforation on occasion in the large number of rats on which we have operated, but in the majority of cases the only departure from the normal is hypertrophy and dilatation of the loop. There is no inflammatory change in the wall of the loop, and the rest of the alimentary tract is macroscopically and microscopically normal. The argentaffin cells are unaffected.

The presence of excess haemosiderin in the spleens of the anaemic animals suggests that there is a haemolytic element in the anaemic, as may be the case in other macrocytic anaemias. No other pathological change was found in the liver or spleen. The result of these studies is therefore essentially a negative one. They indicate that anaemia can develop in rats with intestinal culs-de-sac without the occurrence of any inflammation, ulceration or degenerative change in the alimentary tract.

SUMMARY.

Apart from dilatation and stagnation in the blind loop, no specific gastro-intestinal lesion was discovered in adult albino rats in which macrocytic anaemia had developed as the result of making a cul-de-sac in the small intestine. Haemosiderosis of the spleen was present in every case.

We are indebted to Dr. A. H. T. Robb-Smith for his kind assistance in the preparation and interpretation of the histological material. The expenses of this investigation were partially defrayed by the Medical Research Council.

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FAT EXCRETION IN RATS WITH INTESTINAL CULS-DE-SAC.

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WATSON, Cameron and Witts (1948) described the development of macrocytic anaemia in rats in which a cul-de-sac was made in the small intestine. They also showed that, apart from dilatation and stagnation in the blind loop, the alimentary tract was anatomically and histologically normal (Cameron, Watson and Witts, 1949, 1950). It therefore appeared possible that the anaemia might be caused by an absorption defect similar to that of sprue, and it was decided to determine the percentage of ingested fat which was excreted in the stools in normal rats, and in operated rats with and without anaemia. A rat was not considered to be anaemic until its haemoglobin had fallen to 10·0 g. per 100 ml. or below (normal 13–15 g.) and its peripheral blood showed the characteristic macrocytic picture.

Methods.

The rats were placed in metal metabolism cages over glass funnels, with devices for separating urine and faeces. They were fed outside the cages, in glass bowls, for one hour each morning and 30 minutes each afternoon. The synthetic diet described by Watson, Cameron and Witts (1948) was used. The solid diet mixture was made up in bulk and kept as stock, and portions were mixed with a suitable quantity of water just before use. Each animal was given a weighed amount of food, and the food left was collected, weighed, and the amount consumed was recorded. The animals had unrestricted access to water in the metabolism cages. The operated rats and the normal controls were as nearly as possible of the same age and size, and all the animals received the same treatment, in order to minimize the effect on the results of uncontrollable variations in food and environment.

Any faeces passed into the feeding bowls were added to the main collections. The faeces were allowed to accumulate in the receivers for 4 days after the beginning of the experiments, and the total quantities passed during this period were used for the estimations. The experiments were continued for three 4-day periods, and the total quantities of fat excreted during the 12 days were used to calculate the results. The faecal fat was measured by the method of Fowweather and Anderson (1946). The fat content of the food was estimated by extracting small quantities of food by the same method, and was found to be 2·1 per cent of the wet weight. Two groups of operated animals were used : (i) Anaemic rats (haemoglobin below 10 g. per 100 ml.), (ii) rats which had been operated upon, but which had not developed the anaemia.

Results.

Table I shows that both groups of operated rats excreted significantly greater proportions of ingested fat in the stools than normal animals. Fat absorption was deficient in most of the operated rats, but a small proportion of them developed

TABLE I.—*Showing the Percentages of Ingested Fat Excreted in the Faeces of Normal Rats, operated Non-anaemic Rats, and Operated Anaemic Rats estimated over Periods of Twelve Days.*

Rat No.	Normal rats: Percentage ingested fat excreted.	Operated non-anaemic rats: Percentage ingested fat excreted.	Operated rats with anaemia: Percentage ingested fat excreted.
1	2·5	11·8	3·9
2	2·4	11·0	6·4
3	3·7	9·7	11·0
4	3·6	13·0	22·6
5	3·2	13·0	23·8
6	6·4	15·1	8·6
7	4·4	11·8	17·3
8	8·2	..	21·6
9	7·4	..	22·6
10	5·1	..	11·5
11	6·5	..	26·6
12	7·4	..	..
13	8·4	..	..
14	8·2	..	..
15	13·3	..	..
16	6·2	..	..
17	6·0	..	..
18	5·8	..	..
Mean	6·04	12·2	15·99
Standard error	0·63	0·65	2·40

	Difference of means.	Probability.
Normal rats and operated rats with anaemia	9·95	<·0001 (significant)
Normal rats and operated rats without anaemia.	6·16	<·0001 (significant)
Operated rats with and without anaemia	3·79	=·15 (not significant)

anaemia without showing impaired fat absorption. In this group of animals there did not appear to be any sepsis or haemorrhage to explain the anaemia. Further work is now in progress to see whether treatment of the anaemic animals with folic acid improves the fat absorption, and also to discover at what stage after the operation the absorption defect first appears.

Summary.

The formation of an intestinal cul-de-sac in the rat appears to cause defective absorption of fat, as determined by the ratio of the amount of fat eaten to the amount excreted in the stools, but the anaemia can occur without measurable defect in fat absorption.

We are grateful to Miss Beryl Brinkhurst, who had charge of the animals during these experiments; to Drs. S. T. E. Callender, D. G. Cameron and G. M. Watson for the supply of operated animals; and to Professor L. J. Witts for advice and criticism. Dr. Watson assisted us with the statistical analysis of the figures.

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DIFFUSIBLE ANTIGENS IN STAPHYLOCOCCAL CULTURES.

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BACTERIAL growth in fluid media produces a mixture of many substances, and to demonstrate the toxicity of resulting filtrates or supernatants their actions on the cells or tissues of animals must be investigated. Toxic effects may be due to one or to several substances. The first step in their analysis is to find suitable indicator effects which are capable of quantitative measurement. This has inherent difficulties, as a single product may have several indicator effects; or conversely, a single effect may be due to several different toxins in the same culture filtrate. Quantitative assessment of the effect would then measure only the constituent present in highest titre. Since diffusible bacterial toxins are antigenic, the components of a toxic mixture may be distinguished by quantitative neutralization combined with the use of indicator effects. The analysis is based on the assumption that when a single toxin has two or more indicator effects, the quantitative relationship of the effects should remain constant when measured by antisera prepared against filtrates from different strains of the same species. Should this relationship not hold, the effect must be due to different toxins and multiplicity of toxins is inferred. Though the theoretical basis of this approach is sound and simple, the practical difficulties encountered are considerable. The greater the number of toxins producing multiple indicator effects the more complex the analysis becomes. For this reason the toxins of many organisms have not so far been successfully analysed.

The most complete analysis of bacterial toxins has been achieved with the clostridia. There appears to be a set number of toxins characterizing each species. Not all of these toxins are produced by every member; some may be shared by all virulent members, while others occur in combinations defining individual types. Several types of *Cl. welchii* can be differentiated in this way by their toxin patterns. Furthermore, the various types so defined show well-marked differences in their host range and the types of lesions caused—differences which are not surprising, since toxin production is the key to virulence in these organisms. With staphylococci no such classification is as yet possible. Burnet (1929) found that three activities of staphylococcal filtrates—haemolysis of rabbit cells *in vitro*, necrotic skin lesions in rabbits and lethal effect on intravenous injection—were manifestations of a single antigenic substance. With the description of fresh indicator effects the concept of multiple toxin production by staphylococci has become accepted, and the toxin investigated by Burnet is known as the α haemolysin. It is now known that antigenically different haemolysins (α and β) are formed, but the evidence for the separate existence of α_2, δ and

γ is not conclusive. In addition coagulase, leucocidin, fibrinolysin, enterotoxin, spreading factor, lipase, protease and proinvasin are said to complete the offensive arsenal of staphylococci. These individual effects have been much investigated, but little is known of the pattern in which they occur in human and animal strains. Analogy with the clostridia suggests that toxin patterns may partly determine the nature of the lesion caused and so explain why some strains produce relatively unimportant skin lesions while others are invasive.

Instead of investigating the individual toxic effects of different strains, we set out to demonstrate the antigenically different diffusible substances formed. With this approach we have at present confined ourselves to the investigation of the number of antigens formed by a series of staphylococci of human and animal origin, tested by reference sera prepared against the Wood 46 strain. We hope later to be able to identify the observed antigenic reactions with specific indicator effects. Since the diffusible antigens are not all necessarily toxins, the number of antigens demonstrable may exceed the number of true toxins formed. Moreover, by the technique employed only those antigens will be revealed for which the antiserum used contains sufficient antibody, and other toxins may be present but undetected. In spite of these limitations evidence will be presented that in fact a good correlation exists between the production of diffusible antigens and other criteria of pathogenicity. A plate method described previously (Elek, 1948) offered a means of enumerating the diffusible antigens of staphylococci, and in a later paper (Elek, 1949) various methods were discussed by which individual antigen-antibody lines could be identified with some indicator effects. A modification of the method is now used, and the results are correlated with coagulase production, degree of pigmentation, gelatin liquefaction, mannitol fermentation, penicillin sensitivity and strain origin.

METHODS

Plate technique for the demonstration of diffusible antigens.

The base medium consisted of Difco heart-infusion broth with 0·2 per cent of potassium dihydrogen phosphate (KH_2PO_4) and 0·03 per cent magnesium sulphate ($MgSO_4.7H_2O$). The pH was adjusted to 7·4, and 1·5 per cent New Zealand agar powder was added. After filtration through paper pulp the medium was sterilized by autoclaving at 15lb. per square inch for 15 minutes. Sterile horse blood was centrifuged and the red cells washed three times with normal saline. The red cells were then resuspended to their original volume in saline. A 10 per cent solution of white saponin in distilled water was prepared and sterilized in the autoclave at 10 lb. per square inch for 30 minutes ; one volume of this saponin solution was added to twenty volumes of the reconstituted horse red cells, and the mixture incubated for 15 to 30 minutes to complete lysis. The laked cells were stored in the refrigerator and were found to be satisfactory for several weeks. Flat-bottomed petri dishes of three-and-a-half inch diameter were used. Into each of these 0·2 ml. of the laked red cells and 12 ml. of the base medium were poured. Even mixing was ensured by gentle rotation of the plate. Finally before the agar had set a filterstrip soaked in staphylococcal antitoxin was embedded below the surface in the middle of the plate. The filterstrips measured 60 mm. by 15 mm., and had been sterilized in the hot-air

oven. The antitoxin used was Refined Staphylococcal Antitoxin Globulins (Burroughs Wellcome & Co.,) of a strength of 1250 units per ml. The plates thus prepared were dried for 2 to 3 hours and were used the same day. Not more than four strains were inoculated on each plate in the form of streaks at right angles to the filterstrip. The plates were placed in air-tight jars which were then filled with a mixture of 30 per cent carbon dioxide and 70 per cent air through a reducing valve from a steel cylinder, and incubated at 37° C. for four days ; final readings were taken after a further 48 hours at room temperature.

Coagulase production.

Human citrated plasma from the blood bank was diluted 1 in 10 with sterile nutrient broth immediately before use. A Wassermann tube containing 0·5 ml. of this mixture was inoculated with an entire colony from an overnight blood agar culture. The tubes were placed in a water-bath at 37° C., and readings were made 3 hours later. The negative tubes were then left at room temperature for the final reading after 24 hours. Two control tubes were included with each group, one containing the diluted plasma uninoculated to detect spontaneous clotting, and the other inoculated with a known-coagulase positive strain. Those strains which appeared negative by this technique were checked by using two drops of an overnight broth culture in place of the solid growth.

Pigment production.

Milk agar plates were poured from an autoclaved mixture of one part whole cow's milk and four parts of ordinary nutrient agar. Streak inoculations were made and the plates were incubated in air at 37° C. for 48 hours, and then left at room temperature for a further three days before being read. The following four types of pigmentation were differentiated : albus, off-white, aureus and citreus. The aureus strains were graded arbitrarily + to +++ according to depth of colour.

Mannitol fermentation.

Bijou bottles containing slopes of ordinary nutrient agar incorporating 1 per cent mannitol and Andrade's indicator were inoculated over the surface. They were incubated in air at 37° C. and read up to one week ; the development of a red colour was recorded as positive.

Gelatin liquefaction.

Nutrient broth was made up with 12 per cent Gurr's gelatin and sloped in tubes. Strains were streaked over the surface and left at room temperature for three weeks, readings being taken weekly. Slight pitting around the growth was accepted as positive. Strains found to be negative after this time were incubated at 37° C. in air for a further 48 hours and then placed in the refrigerator to cool ; failure to resolidify was regarded as a positive result. Considerable incubation was required for many of the strains before liquefaction occurred. Incubation at room temperature for three weeks was found to be more sensitive

than incubation for 48 hours at 37° C., as with the former method a small degree of surface-pitting could be demonstrated, while this was missed with the latter procedure.

Penicillin sensitivity.

Strains were streaked over horse blood agar plates, and a filterstrip soaked in penicillin solution (500 units per ml.) was placed at right angles to the centre of the streaks. The Oxford staphylococcus was always included as a control. Sensitivity or resistance was recorded after overnight incubation at 37° C. in air.

MATERIAL.

Three hundred and fifty-nine strains were examined, Of these, 200 were selected from routine human material, and were regarded as pathogens because they came from lesions typically caused by staphylococci. They were mostly isolated in pure primary culture and were all coagulase-positive. Nine strains were from carbuncles, 24 from boils, 21 from breast abscesses, 16 from conjunctivitis in the newborn, 20 from whitlows, 54 from miscellaneous abscesses, 6 from otitis, 6 from osteomyelitis and 10 from post-operative infections. The remaining 34 strains were from a variety of conditions. The second group consisted of 100 unselected strains from the skin and nose of hospital in-patients with no skin or nose lesions. Ninety consecutive patients were swabbed, using a dry swab for both nostrils, and a swab moistened with broth rubbed over an area about the size of a square inch of the upper abdomen. Each swab was inoculated on to a nutrient agar plate and a 7·5 per cent sodium chloride-nutrient agar plate. Of the 100 strains 26 were found to be coagulase-positive. Twenty-one of the coagulase-positive strains were isolated from the nose (23·3 per cent of the patients) and only 5 (5·5 per cent) from the skin. All the strains obtained from lesions and from carriers were tested either within a few days of isolation (without subculturing), or they were freeze-dried forthwith and tested immediately after reconstitution. A further 59 strains were from animal lesions. These were not freshly isolated strains, but were all coagulase-positive.

RESULTS.

The serum used throughout these experiments was one which had been prepared against the Wood 46 strain, and when this organism was tested by the technique described, it was found to produce no fewer than ten lines. A line drawing of the pattern obtained, with the Wood 46 uppermost and another strain below, is shown in Fig. 1, while a photograph of the typical appearances given by two different strains is shown in Fig. 2. Staphylococci other than the Wood 46 generally produced fewer lines. The maximum number of lines formed was constant for each strain, though their position relative to each other was somewhat variable. Most of the lines appeared within 24 to 48 hours, but some appeared only after several days. The early lines increased in density and length with incubation and were easily seen; the later lines were much finer, and occasionally they were difficult to see. Sometimes a narrow zone of turbidity was formed which later differentiated into fine lines.

Coagulase production.

The most striking experimental finding was the complete agreement between coagulase activity and line production. All the coagulase-positive strains from human sources flocculated with the Wood 46 antitoxin, whereas none of the coagulase-negative strains did so (Table I). In the animal pathogens there is also a close correlation between coagulase production and the ability to produce lines, but the agreement is not absolute. Coagulase-positive skin strains produce fewer lines than the proved pathogens, the difference being highly significant statistically. Moreover, there is a highly significant difference between the average number of lines produced by the human and animal pathogens.

TABLE I.—*Comparison of Line Production by Strains Obtained from Various Sources.*

Group.	Number of strains examined.	Number of strains producing lines.	Mean number of lines.
Coagulase-positive human pathogens	200	200	6·37
Coagulase-positive animal pathogens	59	55	4·69
Coagulase-positive skin and nose strains	26	26	5·19
Coagulase-negative skin and nose strains	74	none	*nil*

Type of lesion.

When the human pathogens are grouped according to the lesions from which they were isolated there is seen to be no statistically significant difference in the number of lines produced by the different groups (Table II).

TABLE II.—*Comparison of Line Production in Strains from Different Lesions.*

Lesion.	Number of strains.	Mean number of lines.
Breast abscesses	21	6·33
Boils	24	6·12
Carbuncles	9	6·00
Various abscesses	54	6·61
Osteomyelitis	6	6·00
Conjunctivitis of infants	16	6·69
Otitis	6	6·66
Postoperative wound sepsis	10	6·30
Whitlows	20	6·25
Unclassified lesions	34	6·24

Penicillin resistance.

Strains showing resistance to penicillin do not produce fewer lines than those which are sensitive. Of 183 human pathogens examined 131 (71·5 per cent) were sensitive, the average number of lines produced being 6·34. The 52 penicillin resistant strains averaged 6·60 lines per strain. This difference is not statistically significant.

Pigment production.

The incidence of various degrees of pigmentation in the groups investigated is shown in Table III. There is a striking difference in the distribution of the various grades of pigmentation between the coagulase-positive and coagulase-negative groups. This is shown graphically in Fig. 3. In the coagulase-positive

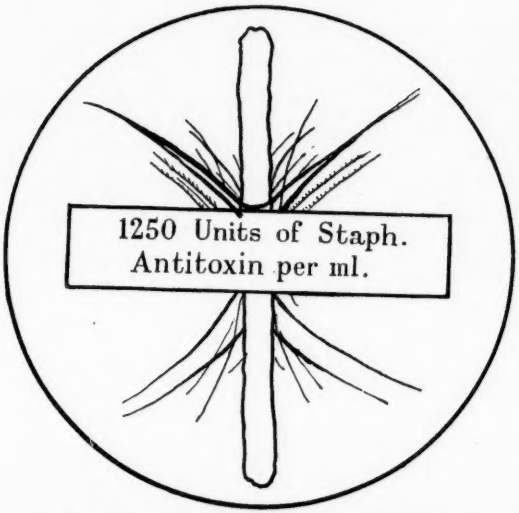

FIG. 1.—A line drawing of the patterns obtained with two different strains of staphylococci; the uppermost strain is the Wood 46.

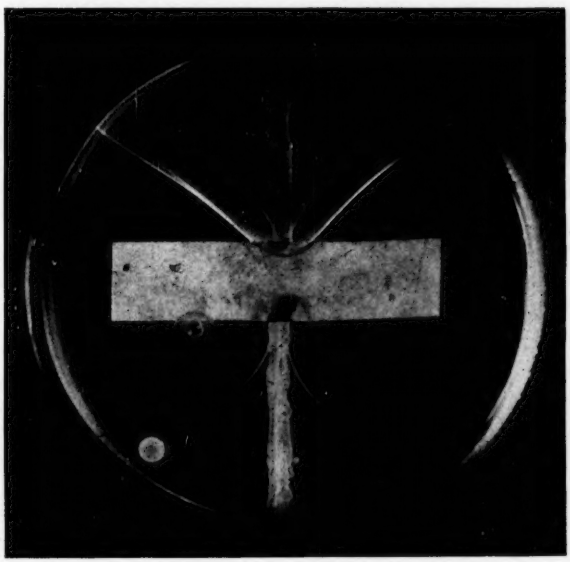

FIG. 2.—A photograph showing the patterns obtained with two different strains of staphylococci.

TABLE III.—*The Relationship between the Degree of Pigmentation and Line Production.*

Group.	Number of strains.	Albus.		Off-white.		Aureus +.		Aureus ++.		Aureus +++.	
		Number of strains.	Mean number of lines.	Number of strains.	Mean number of lines.	Number of strains.	Mean number of lines.	Number of strains.	Mean number of lines.	Number of strains.	Mean number of lines.
Coagulase-positive	281	21	4·10	22	5·45	69	5·81	97	6·29	72	6·15
Coagulase-negative	72	60	*nil*	3	*nil*	6	*nil*	3	*nil*	*nil*	*nil*

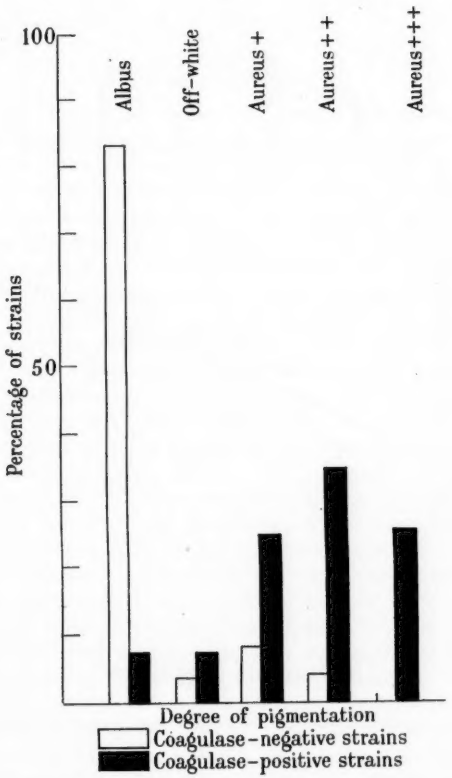

FIG. 3.—Graph illustrating the distribution of the various degrees of pigmentation among 281 coagulase-positive strains and 72 coagulase-negative strains.

group the number of lines produced can be correlated with the degree of pigmentation. There is a highly significant increase in the number of lines as colour deepens from albus to aureus +++. This can be shown by plotting the five categories of pigmentation against the mean number of lines produced. The

best fitting straight line—representing the regression of number of lines on grade of colour—passes through 4·81 for albus and 6·48 for aureus +++ (Fig. 4).

Mannitol fermentation.

In a group of 246 pathogenic strains most were mannitol-positive (Table IV). Among the animal pathogens no significant difference in line production was found between the positive and negative strains. The group of carrier strains was too small for analysis, but there appears to be a significant difference in line production within the group of human pathogens.

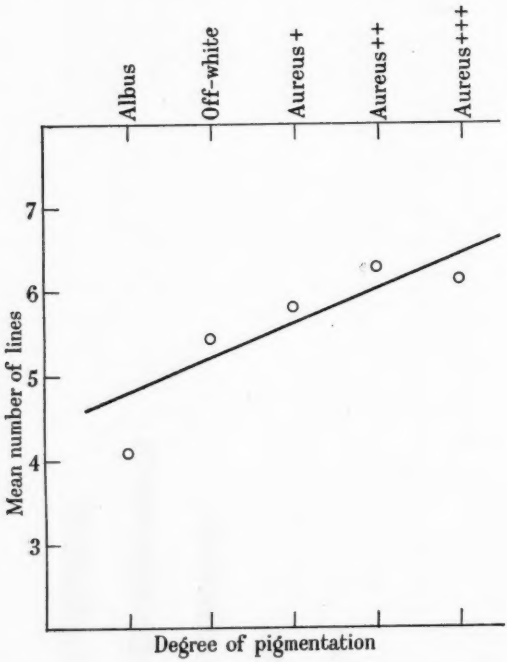

FIG. 4.—Graph showing the increase in the mean number of lines with increasing degree of pigmentation.

TABLE IV.—*Relation of Line Production to Mannitol Fermentation.*

Strains.	Total numbers examined.	Mannitol fermentation.		Mean number of lines.	
		+	−	Mannitol +.	Mannitol −.
Human pathogens	188	182	6	6·41	4·50
Animal pathogens	58	46	12	4·60	4·66
Coagulase-positive carrier strains	15	13	2	6·08	3·00

Gelatin liquefaction.

Of 195 coagulase-positive strains only 123 were gelatin liquefiers (63 per cent). There is a reduction in the mean number of lines produced by gelatin-negative strains in both the human and animal groups of pathogens (Table V).

TABLE V.—*Relation of Gelatin Liquefaction to Lines.*

Strains.	Total number examined.	Gelatin		Average number of lines.	
		+	−	Gelatin +.	Gelatin −.
Human pathogens	165	99	66	7·33	6·69
Animal pathogens	27	22	5	5·63	3·00
Coagulase-positive carrier strains	3	2	1	7·50	9·00

DISCUSSION.

It must be emphasized that when any strain is tested against a reference serum the number of flocculation lines demonstrable cannot exceed that of the constituent antibodies. In this work the number of the lines was limited by the diffusible antigens formed by the Wood 46 strain. Though this is generally regarded as a representative example of toxicogenic staphylococci, it is now known not to produce certain toxins, notably the enterotoxin and the β-haemolysin. It is not suggested that all the lines produced can be identified with toxins, but with suitable sera the recognition of the α- and β-haemolysins is relatively easy. In this connection it may be noted that analogous work with diphtheria bacilli (Elek, 1948; Ouchterlony, 1948) has shown that apart from the diphtheria toxin, which is believed to be a single entity, at least two other diffusible antigens having no lethal or dermotoxic action can be demonstrated after prolonged incubation. Similar work with strains of *Cl. welchii*, however, showed a correlation between the number of lines produced and the number of toxins known to be associated with various types (Elek, 1949). In the present work, since the antiserum was prepared against a representative toxicogenic strain, the majority of its antibodies are likely to be antitoxins; consequently the formation of flocculation lines is most probably an index of toxicogenicity.

While there is complete agreement between coagulase activity and line production amongst human pathogens, there is a slight discrepancy in the animal group. In this connection it should be stressed that the animal strains were not freshly isolated, and loss of toxicogenicity might have occurred. There are three possible explanations to account for the difference between the average number of lines produced by the human and animal pathogens. Firstly as a result of repeated subculturing not only did four of the strains lose completely the ability to produce toxin, but there might have been a reduction in the number of toxins in the remainder. Secondly, the animal strains may produce toxins different from those of the human strains, and not neutralized by the antitoxin used. This would result in an apparent reduction of the number of lines. Thirdly, it is possible that animal strains normally produce fewer diffusible antigens. It is known, for example, that animal strains of staphylococci generally fail to produce fibrinolysin. The fact that skin strains produce fewer lines than those obtained from lesions suggests that they produce fewer toxins. If this were so, coagulase production would slightly overestimate the potential pathogens amongst skin strains. It must be remembered that coagulase testing is based on the

observation that all strains from lesions are positive ; from this it has been inferred that all coagulase-positive strains are potentially pathogenic. As yet there is no simple means of assessing the pathogenicity of coagulase-positive strains obtained from sources other than lesions. It may well be that routine coagulase testing of carrier strains—although detecting all the pathogens—may in fact include some that are less likely to produce human lesions.

The absolute agreement between line production and coagulase formation in human strains and the similar agreement for animal strains are of great interest, for the association between coagulase production and pathogenicity is now well established. The fact that virulence of staphylococci is linked with coagulation of plasma and the ability to produce toxin must be more than co-incidence. Indeed the question of virulence as applied to staphylococci requires clarification. MacLeod and Pappenheimer (1948) defined pathogenicity as the capacity of micro-organisms to produce disease, and virulence as a measure of pathogenicity. Further, they state that diseases due to bacteria fall into three groups : in the first toxin production is the major factor, invasion being absent ; in the second group the organism is invasive without producing toxin ; and in the third both toxin production and invasion contribute to virulence. Blair (1948) classes staphylococci in the third group. The pathogenicity of any strain in his view represents the total effect of both factors working together at times one or the other being predominant. In some cases exotoxin is undoubtedly responsible for the manifestations of disease, but more often invasion of the tissues is the main feature. He concludes that " a small number of pathogenic staphylococci produce either no exotoxin or only an insignificant amount ; infections due to these non-toxicogenic organisms are referable to their invasive capacity." Wilson and Miles (1946) on the other hand suggest that it is better to retain the term " virulent " in its correct sense of poisonous, without any implication as to how the poisonous effect is produced. In their view " no mechanical theory of the pathogenic action of bacteria is compatible with our knowledge of the way in which the tissues deal with inert particles which had gained access to them. The basis of all harmful effects of bacterial infection is quite certainly chemical . . . " The two views are not necessarily contradictory ; the latter attempts to explain in chemical terms the mode of invasiveness. It places on the broadest possible basis our concept of toxins. Nevertheless the experimental findings sharply contradict Blair's statement that non-toxicogenic strains may produce disease. Amongst 200 strains examined from lesions not one was found to lack toxin production, in spite of the artificial limitations set by the use of a single reference serum.

The failure to find any difference in the number of lines between strains obtained from different human lesions bears out the view that as regards toxin patterns there is no difference between strains causing relatively mild surface lesions and those causing more serious infections. How far quantitative differences in toxin production come into play cannot, of course, be assessed by the method employed.

As it is common experience that penicillin-resistant strains are as virulent as sensitive ones (Spink, Hall and Ferris, 1945 ; North and Christie, 1946), it is not surprising to find that they fail to show a reduction in the number of lines. It is interesting to note, however, that the ratio of sensitive to insensitive strains varied according to the lesion. Taking conjunctivitis of infants and postoperative

sepsis as a group representing hospital infections (26 strains), the ratio of sensitive to resistant strains was 0·4 to 1, whereas in the remainder of the infections the ratio was 3·5 to 1. It seems therefore that lesions which apparently originated in hospital are associated with a higher incidence of penicillin-resistant strains than those arising outside (Barber and Rozwadowska-Dowzenko, 1948; Martyn, 1949; Forbes, 1949; Barber, Hayhoe and Whitehead, 1949; Rountree and Thomson, 1949, and Cairns and Summers, 1950).

Although pigment production is no longer used as a criterion of pathogenicity, it is interesting to note that the greater the degree of pigmentation the more lines are produced. This appears to be in good agreement with the old observation that the deeply pigmented strains are more likely to be pathogenic. Lack of mannitol fermentation and of gelatinase production in human pathogens appear to be associated with the production of fewer lines.

SUMMARY.

An experimental technique based on the double diffusion gradient principle designed to reveal different diffusible antigens in staphylococcal plate cultures is described. Flocculation is observed in the form of lines which permit an assessment of the number of antigens produced by different strains. A single representative antitoxin prepared against the Wood 46 strain showed that antigens were produced by all coagulase-positive strains but none by coagulase-negative staphylococci in a group of 359 strains examined. This absolute correlation is brought forward as evidence that the production of diffusible antigens is a *sine qua non* of virulence. Although it is not claimed that each flocculation line produced represents a toxin, it is certain that every diffusible toxin will yield a line in the presence of sufficient antibody. The number of lines is therefore likely to be a rough measure of the toxin pattern.

The mean number of lines produced by animal pathogens was significantly lower than that produced by the human pathogens. Coagulase-positive skin strains also produced fewer lines than strains from lesions. Possible explanations for these differences are discussed.

Lesion of origin and penicillin-resistance have no correlation with line production. Increasing degrees of pigmentation, mannitol fermentation and gelatinase production are associated with a greater number of lines.

There is no evidence that invasive staphylococci may fail to produce exotoxin.

We wish to express our sincere thanks to Dr. J. A. Fraser Roberts, of the Department of Medical Statistics, London School of Hygiene and Tropical Medicine, for kindly carrying out the statistical analysis of our results. Our thanks are also due to Professor T. Crawford for helpful criticism, and for assistance in the preparation of this paper.

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THE ACTION OF SULPHANILAMIDE ON BACTERIOPHAGES T_1–T_7.

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THE influence of sulphanilamide on bacteriophage multiplication has been studied on several occasions without success, presumably because multiplication of the host-bacteria was inhibited (Delbrück and Luria, 1944; Wahl, Nitti and Faquet, 1946).

In our studies on the action of sulphonamides we have been able to obtain good growth of *Escherichia coli* in media containing 2000 mg./l. of sulphanilamide by the addition to the medium of the five non-competitive sulphanilamide antagonists (Winkler and de Haan, 1948). This opened the possibility of studying the influence of high concentrations of sulphanilamide on bacteriophage multiplication, without interfering with bacterial growth. The results of these experiments are reported here.

MATERIALS AND METHODS.

Through the courtesy of Prof. Delbrück we were able to use *Escherichia coli* B and the bacteriophages T_1–T_7 of Demerec and Fano (1945).

Escherichia coli was cultured in a synthetic medium (medium A) containing per litre: Glucose, 2 g.; NH_4Cl, 0·2 g.; KH_2PO_4, 0·6 g.; K_2HPO_4, 0·6 g., and $MgSO_4$, 0·05 g.; pH, 6·6. The medium was completely free of *p*-aminobenzoic acid. The non-competitive sulphanilamide antagonists, methionine, xanthine, thymine and valine were each added at a concentration of 30 mg./l. (medium B); in some instances serine was added at a concentration of 20 mg./l.

Bacterial counts were made by the technique of Julius (1938), which is a modified plating method and has given reliable results in our previous work (Julius, 1941, 1942; Havinga, Julius, Veldstra and Winkler, 1946). In preliminary experiments bacterial growth and lysis were measured by turbidity with a Moll extinctiometer, as is usual in our laboratory.

Bacteriophages were prepared by inoculating the basal medium with 10^7 viable bacteria and 10^5 bacteriophage particles per ml., and filtering after lysis had occurred through G5 on 3 sintered glass filters. The filtrate generally contained 10^8 to 10^9 bacteriophage particles per ml. Plaque counts were made with Gratia's (1936) double layer plate method according to the modification of Hershey, Kalmanson and Bronfenbrenner (1943). In experiments with bacteriophage T_4, 20 μg. tryptophan per ml. was added.

One-step growth curves were obtained by the technique of Ellis and Delbrück (1939). Adsorption mixtures, unless stated otherwise, contained between 0·5 and 2×10^8 bacteria and about 1 to 5×10^7 bacteriophage particles. Adsorption

and dilution tubes were mixed by continuous aeration. Eight minutes after adsorption decimal dilution tubes down to 1 in 100,000 were prepared. The number of free bacteriophage particles was estimated after centrifugation of the 1 in 100 dilution at 10,000 r.p.m. About 90 per cent adsorption was generally obtained (Table II). The dilution tubes 1 in 1000 and 1 in 100,000 were used as growth tubes, and the number of plaque-forming particles estimated at frequent intervals with the double layer plates. Burst sizes varied from 60 to 160.

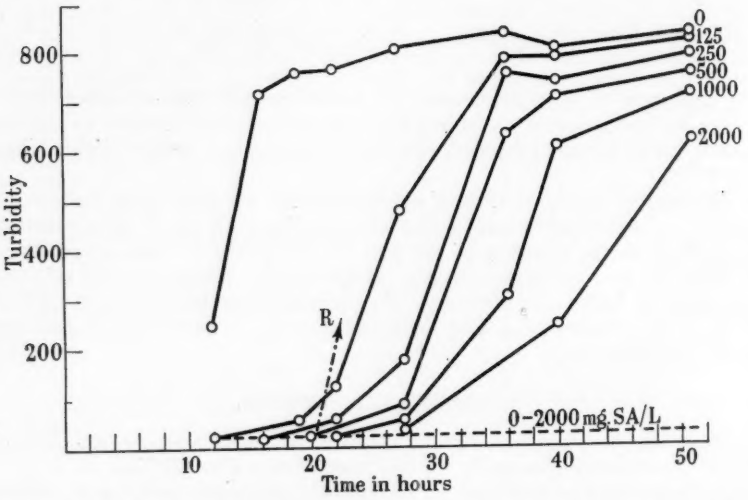

FIG. 1.—Influence of varying concentrations of sulphanilamide on bacteriolysis of *Escherichia coli* B by bacteriophage T_1.

——— Without bacteriophage.
- - - - - - With bacteriophage T_1 (all cultures are lysed).
—·—·—·— Resistant growth in lysed culture.

RESULTS.

Preliminary experiments.

Non-competitive sulphonamides antagonists for E. coli *B.—E. coli* B behaved in general like our *E. coli* (Strain 2) studied previously (Winkler and de Haan, 1948). Methionine, xanthine, thymine and valine seem to be the end-products of the reactions blocked by sulphanilamide. By the addition of these four non-competitive antagonists normal growth was obtained with *E. coli* B in the presence of as much as 2000 mg. sulphanilamide/l. The growth rate was decreased to a greater extent, however, than with *E. coli* (Strain 2), especially with small inocula. A further difference between the two strains was that serine did not act as a non-competitive antagonist for *E. coli* B. These features did not affect the principle, however, and it remained possible to study the action of high sulphanilamide

concentrations on bacteriophage multiplication without unduly reducing bacterial growth.

Inhibition of bacteriolysis by sulphanilamide.

Tubes containing varying concentrations of sulphanilamide (0, 125, 250, 500, 1000 and 2000 mg./l.) in a medium with non-competitive sulphanilamide antagonists (medium B) were inoculated with 3×10^6 bacteria or with both bacteria and 3×10^4 bacteriophage particles. In the case of phage T_4 and T_6 20 μg./l. tryptophan was added. Turbidities were estimated at intervals.

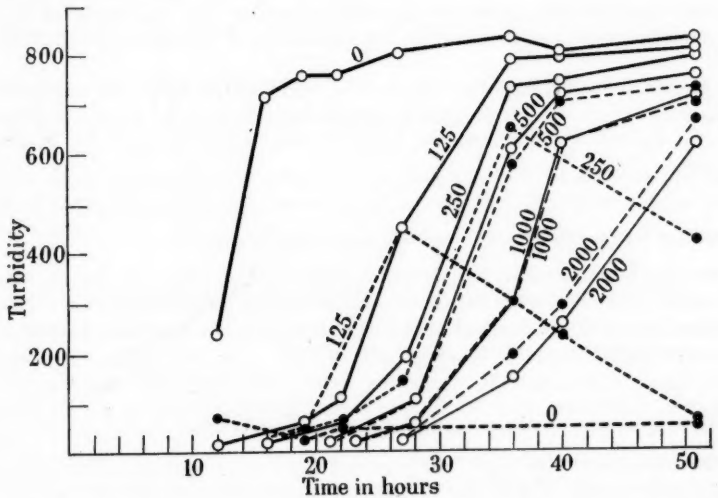

FIG. 2.—Influence of varying concentrations of sulphanilamide on bacteriolysis of *Escherichia coli* B by bacteriophage T_2.

——— Without bacteriophage. - - - - - - - With bacteriophage.

Results with bacteriophage T_1 are given in Fig. 1. In the controls without bacteriophage sulphanilamide reduced the growth-rate in spite of the presence of the five antagonists. It should be realized, however, that without non-competitive antagonists about 60 mg./l. sulphanilamide suppressed visible growth completely. All cultures to which bacteriophage T_1 was added were lysed. In the control tube without sulphanilamide a delayed growth was observed due to a resistant *E. coli*. It was omitted in the figure.

The influence of sulphanilamide on lysis by bacteriophage T_2 is shown in Fig. 2. Cultures without bacteriophage showed growth as before. With bacteriophage the result was entirely different. The culture with bacteriophage, but without sulphanilamide, showed complete lysis. In the presence of 125 or 250 mg./l. sulphanilamide, lysis was less complete. Above 500 mg./l. sulphanilamide

no lysis occurred and bacterial growth equalled that of cultures without bacteriophage. This growth is not due to bacteriophage-resistant bacteria.

Analogous results were obtained with the bacteriophages T_4 and T_6, whereas no such lysis-inhibition by sulphanilamide was observed with the bacteriophages T_1, T_3 and T_7.

The effect described was obtained with varying inocula of both bacteriophage and *E. coli* B. Although the inhibition of bacteriolysis was more pronounced with small inocula (presumably by delayed bacteriophage adsorption) it was always present.

The effect was not due to an increase in generation time induced by sulphanilamide. By culturing *E. coli* at lower temperatures it was shown that an increase in generation time in itself did not affect bacteriolysis. Again, a decrease of the generation time in the presence of sulphanilamide by the addition of caseinhydrolysate even seemed to enhance the inhibition of bacteriolysis by sulphanilamide.

All causes for an unspecific effect thus being eliminated, we concluded that high concentrations of sulphanilamide inhibit bacteriolysis of *E. coli* B by bacteriophages T_2, T_4 and T_6, whereas the lysis by the bacteriophages T_1, T_3 and T_7 is unaffected.

Inhibition of bacteriophage multiplication by sulphanilamide.

It was soon established by plaque counts that in the cultures in which sulphanilamide inhibits bacteriolysis no bacteriophage multiplication takes place, whereas in the control tubes without sulphanilamide the number of bacteriophage particles increased from 10^3 to about 10^8 or 10^9.

Mechanism of action of virus inhibition by sulphanilamide.

In the chemotherapy of viruses such as the bacteriophages, there are at least three possible points of attack: (*a*) extracellular inactivation of virus particles, (*b*) inhibition of adsorption of virus to host cells, and (*c*) intracellular inhibition of virus multiplication.

To ascertain with which stage of the life-cycle of the bacteriophage sulphanilamide interferes, we first studied the effect of 2000 mg./l. sulphanilamide on free T_4 bacteriophage particles (Table I) and on adsorption (Table II). With bacteriophage T_2 analogous results were obtained. It is evident from the results shown in

TABLE I.—*Survival of Free Bacteriophage T_4 in 2000 mg./l. Sulphanilamide.*

Time in hours.	Number of bacteriophage particles × 10^8 at 0.	24.	48.
Medium A . .	0·81	0·22	0·26
Medium B . .	0·81	0·36	0·33
Medium B + 2000 mg./l. S.A. . . .	0·81	0·26	0·26

S.A. = Sulphanilamide.

TABLE II.—*Influence of Sulphanilamide and of Bacteriophage T_4 on* Escherichia coli *B.*

Growth in medium.	Adsorption in medium.	Viable bacteria ml. ($\times 10^7$).	Bacteriophage particles/ml. ($\times 10^7$).	Per cent adsorption in 8 min.
A	A	6·2	1·2	84
A	A	3·2	2·0	84
A	A+2000 mg./l. S.A.	23	0·32	94
..	A	23	0·32	96
A	B+2000 mg./l. S.A.	8	2·1	95
..	A	8	2·1	95
A	B	6·2	1·75	95
..	A	6·2	1·75	95
B+2000 mg./l. S.A.	B+2000 mg./l. S.A.	10	4·9	93
..	B+2000 mg./l. S.A.	6·6	4·6	91

S.A. = Sulphanilamide.

the tables that sulphanilamide does not inactivate free bacteriophage particles and that sulphanilamide does not inhibit the adsorption of bacteriophage particles to *E. coli* B.

The effect of sulphanilamide on intracellular bacteriophage multiplication.

The only reliable method of showing the influence of a drug on intracellular bacteriophage multiplication is the one-step growth technique in which one life cycle of the virus is singled out. Any inhibition of intracellular multiplication will result in an extension of the latent period or in a reduction of the mean number of bacteriophage particles liberated per bacterium.

One-step growth curves were obtained with bacteriophage T_4 in medium A or B, or without sulphanilamide, using *E. coli* B grown in medium A as an inoculum. With sulphanilamide a very slight increase in the latent period was often observed, but the burst size was never affected. Using bacteria grown in medium A, sulphanilamide had no direct effect on the multiplication of bacteriophage T_4 in one-step growth.

The delayed effect of sulphanilamide.

The above effect was not unexpected. Bacteriophage multiplication might be inhibited by interference with a supposed phage metabolism, although it is generally agreed that bacteriophage multiplication is largely dependent on bacterial metabolism (anabolism). Bacteriophage multiplication might thus be inhibited by interference with aspects of bacterial metabolism not affecting bacterial growth. For instance, inhibition of a surplus production by certain enzyme systems might inhibit virus multiplication without affecting bacterial growth. Again some enzyme systems might be essential for phage multiplication, but not essential for bacterial growth.

The above results exclude any direct action on bacteriophage metabolism. Yet the action of sulphanilamide on bacterial growth is generally delayed, i.e.

only becomes evident after one or two normal divisions. Presumably the end-products of the inhibited enzymes are still present in sufficient amount to permit one or two divisions. Now in " one-step growth " experiments bacteriophage is adsorbed onto the bacteria within a few minutes, and the host cell is infected before the sulphanilamide effect can develop. Inhibition of bacterial metabolism might well be without effect on bacteriophage reproduction unless sulphanilamide was added before the addition of bacteriophage.

" One-step growth " experiments were set up as before, using as inoculum *E. coli* B grown in medium B with 2000 mg./l. sulphanilamide. In this case a complete suppression of bacteriophage multiplication (T_4) was observed when 2000 mg./l. sulphanilamide was added in the one-step experiment (Fig. 3).

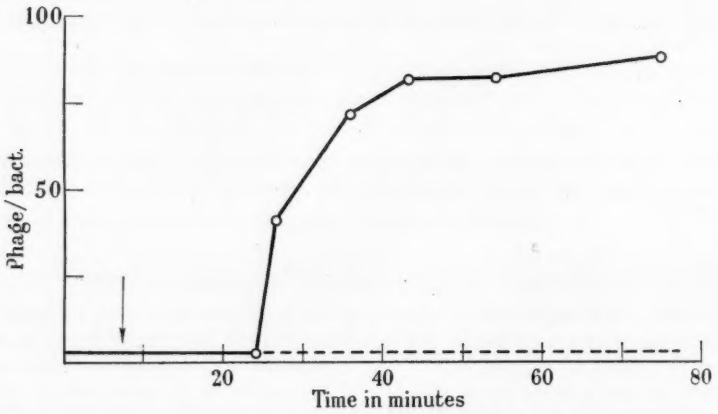

FIG. 3.—Influence of sulphanilamide on growth of bacteriophage T_4. Abscissa, time in minutes. Ordinate, number of bacteriophage particles per bacterium.

Curve 1 ------ multiplication of bacteriophage T_4 in bacteria grown in medium B *plus* 2000 mg./l. sulphanilamide.

Curve 2 ———— Control, bacteria grown either without sulphanilamide or non-competitive antagonists (= medium A), or grown in medium A *plus* 12 mg./l. sulphanilamide to obtain the same growth rate as in the culture producing the host bacteria for Curve 1.

This effect was not due to a reduced growth rate in the culture from which the host cells were derived. By heavy inoculation the growth rate in sulphanilamide *plus* non-competitive antagonists was made equal to the control. Various other experiments showed beyond doubt that even in experiments in which the growth rate of the culture from which the host cells were obtained had been reduced, this reduction did not affect bacteriophage multiplication. The bacteriophage multiplication is shown in Fig. 3. It is evident that in bacteria cultured in 2000 mg./l. sulphanilamide (with non-competitive antagonists), bacteriophage T_4 cannot multiply. That this effect is indeed specific is shown by the fact that reproduction of bacteriophage T_1 was not inhibited under the same conditions. We thus conclude that in bacteria grown in the presence of sulphanilamide and non-competitive antagonists, intracellular bacteriophage reproduction (T_4) is inhibited.

Bacterial enzymes inhibited in the range of sulpahnilamide concentration which inhibits bacteriophage.

The experiments reported in the previous section show that sulphanilamide acts by interfering with bacterial metabolism, as it is the growth of the host cells in media containing sulphanilamide which is essential for the effect. No non-specific sulphanilamide effect is involved as bacteriophage reproduction is re-established by the addition of *p*-aminobenzoic acid. Some p-aminobenzoic acid-dependent reaction is thus involved.

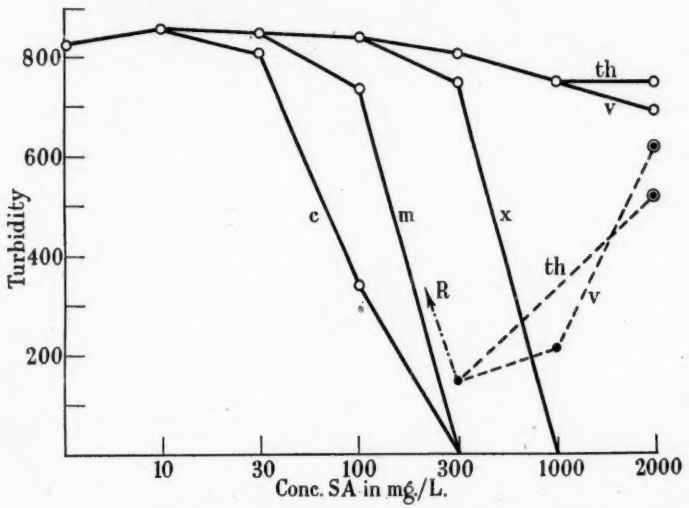

FIG. 4.—Influence of non-competitive antagonists on sulphanilamide inhibition of growth of *E. coli* B, with and without bacteriophage. Abscissa, concentration sulphanilamide in mg./l. Ordinate, turbidity.

———— Controls without bacteriophage.
c = Medium A.
m = Medium A + methionine.
x = Medium A + methionine + xanthine.
th. = Medium A + methionine + xanthine + thymine.
v = Medium A + methionine + xanthine + thymine + valine.
- - - - - - - With bacteriophage.

With bacteriophage the cultures c, m and x are completely lysed (not shown in fig.) or show resistant growth (R). Only on the addition of thymine and valine does growth occur. It was assured that this growth was not bacteriophage-resistant.

The activity of sulphanilamide against virus reproduction might then be due to the inhibition of one of the four *p*-aminobenzoic acid-dependent enzyme systems leading to methionine, xanthine, thymine and valine, or to an interference with some as yet unknown function of *p*-aminobenzoic acid. Which of the four enzyme systems of the former possibility is involved might be studied by comparing the sulphanilamide concentration at which each system is inhibited with the concentration at which bacteriophage inhibition occurs.

To this end *E. coli* B was cultured in medium A with increasing concentrations of sulphanilamide in five series of tubes. The first series acted as control. Methionine was added to the second, methionine and xanthine to the third. The fourth series contained methionine, xanthine and thymine. The fifth also valine, thus containing medium B. To a duplicate set of tubes bacteriophage was added. At regular intervals turbidity readings were taken. Fig. 4 shows the results at 48 hours. In the series without bacteriophage a step-wise increase in the tolerated sulphanilamide concentration was observed, as in previous work

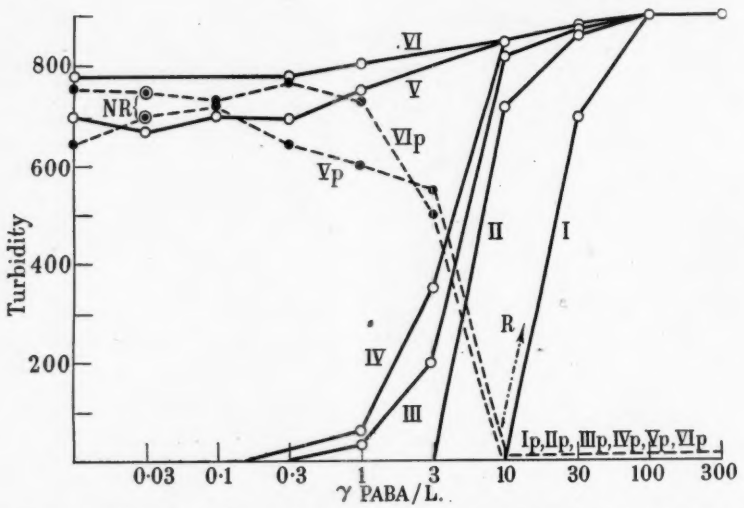

FIG. 5.—Influence of non-competitive sulphanilamide antagonists on the growth of *E. coli* in the presence of sulphanilamide and *p*-aminobenzoic acid, with and without bacteriophage. Abscissa, concentration *p*-aminobenzoic acid in μg./l. Ordinate, turbidity. Constant sulphanilamide concentration 2000 mg./l. Bacterial growth at 48 hours.

——— Control without bacteriophage. - - - - - - - - With bacteriophage (p).

I,	Ip	medium	A.						
II,	IIp	,,	A	with	methionine.				
III,	IIIp	,,	A	,,		and	xanthine.		
IV,	IVp	,,	A	,,		,,		and	serine.
V,	Vp	,,	A	,,		,,		,,	and thymine.
VI,	VIp	,,	A	,,		,,		,,	and valine.

With bacteriophage all cultures show lysis, except Vp and VIp. Resistant growth was omitted from the figure and only indicated as R.

(Winkler and de Haan, 1948). With bacteriophage all cultures showed lysis, until thymine was added. On the addition of thymine high sulphanilamide concentrations inhibited bacteriophage multiplication (lysis).

The experiment was varied by culturing *E. coli* in five series of tubes all containing 2000 mg./l. sulphanilamide. Varying concentrations of *p*-aminobenzoic acid were added to each series and the same concentrations of non-competitive antagonists as before were used. The results are represented in

Fig. 5. Without bacteriophage the addition of each additional non-competitive antagonist decreased the concentration of *p*-aminobenzoic acid which supported bacterial growth, until with all four non-competitive antagonists growth without *p*-aminobenzoic acid was obtained.

On the addition of bacteriophage all cultures were lysed until thymine was added. Again, as soon as the *p*-aminobenzoic acid concentration fell below the level which antagonizes inhibition by sulphanilamide of the thymine-synthesizing system (10 μg./l.)—that is as soon as this system is inhibited, and the bacteria can only grow by the addition of thymine—bacteriophage inhibition began.

In both experiments bacteriophage inhibition began as soon as the thymine-synthesizing system was inhibited. This might be a coincidence, bacteriophage multiplication being inhibited at the same concentration of sulphanilamide as the thymine-synthesizing enzyme, or might indicate a narrower relationship; the thymine supply might be sufficient for bacterial growth but insufficient for bacteriophage synthesis or the (still inhibited) thymine-synthesizing enzyme itself might be involved in bacteriophage synthesis. The same reasoning might be applied to valine.

We conclude that the action of sulphanilamide on bacteriophage reproduction is not due to inhibition of the methionine and xanthine synthesizing system. Only the synthesis of thymine and valine or further unknown systems are involved.

Search for substances which antagonize the sulphanilamide effect on bacteriophage.

Whether some unknown function of *p*-aminobenzoic acid was involved in the inhibition of bacteriophage multiplication was studied by trying to find substances which antagonize the sulphanilamide effect on bacteriophage non-competitively. The following substances were tested: Various inorganic salts, ascorbic acid, eight vitamins of the B group, yeast autolysate, adenosinetriphosphoric acid, all available keto acids, twenty-two amino-acids, the peptides valyl-glycine, alanyl-glycine, leucylglycine, glycineanhydrid, glycylglycine, alanylglycylglycine, leucyl-glycylglycine and casein hydrolysate. Furthermore, creatine, sarcosine, glycocyamine, hippuric acid, thymine, thymidine, guanosine, uracil, ribonucleic acid and desoxyribonucleic acid were added.

The bacteriophage inhibition by sulphanilamide was only antagonized by glycine and the glycine-containing peptides. Even with these substances the effect was very slight and not sufficient to warrant any conclusion.

DISCUSSION.

The above experiments show that the intracellular multiplication of the bacteriophages T_2, T_4 and T_6 on *E. coli* B can be inhibited by high concentrations of sulphanilamide, when the interference with bacterial growth is antagonized by the addition of four non-competitive antagonists. The specificity of this effect is stressed by the fact that multiplication of the bacteriophages T_1, T_3 and T_7 is unaffected by sulphanilamide. It was further shown that sulphanilamide does not interfere with an eventual bacteriophage metabolism, but that the effect is due to some interference with bacterial metabolism. The effect must be due to some *p*-aminobenzoic acid-dependent system.

Some digression into our present views (Winkler and de Haan, 1948) on sulphanilamide action may be allowed here. We suppose that sulphanilamide interferes with the synthesis from *p*-aminobenzoic acid of several substances which act as, or form part of, prosthetic groups for the enzymes synthesizing methionine, xanthine, serine, thymine and valine from their precursors. In some instances pteroylglutamic acid is one of these prosthetic groups (probably synthesizing thymine), but in *E. coli* B there is no evidence for this.

In our present case the bacteria are able to grow in the presence of high concentrations of sulphanilamide after the addition of the four non-competitive antagonists. In these bacteria the enzymes synthesizing methionine, xanthine, thymine and valine do not function (possibly because the prosthetic group is missing), but growth is permitted by the supply of the end-products.

The activity of sulphanilamide against virus reproduction might then be due to at least four causes :

1. One or more of the four enzymes leading to methionine, xanthine, thymine and valine are involved :

   (*a*) The supplied concentration of the four end-products is sufficient for bacterial reproduction, but insufficient for the more exacting bacteriophage multiplication.

   (*b*) The presence of the intermediate products derived from *p*-aminobenzoic acid (prosthetic groups ?) is essential for bacteriophage reproduction as the bacteriophage uses them for its own purposes, for instance on apo-enzymes of its own.

   (*c*) The presence of one of the four supposed bacterial apo-enzymes is essential for the bacteriophage. In bacteria grown in sulphanilamide these apo-enzymes might not be available but blocked by compounds containing sulphanilamide (e.g. folic acid analogous with sulphanilamide instead of *p*-aminobenzoic acid).

2. Other enzymes, dependent on intermediate products derived from *p*-aminobenzoic acid and the end-products of which are not yet known, are involved.

It was established that a fourfold increase in the concentration of the four non-competitive antagonists does not re-establish bacteriophage multiplication; possibility 1(*a*) is thereby excluded.

It was shown that the methionine and xanthine-synthesizing systems are not involved. There is a probability that the thymine and valine synthesizing systems are involved, but other unknown systems could not be completely excluded.

Definite evidence on this subject might be obtained if it could be shown that a *p*-aminobenzoic acid-less *E. coli B* mutant strain, grown without *p*-aminobenzoic acid on a medium containing methionine, xanthine, thymine and valine was bacteriophage-resistant. Such an experiment would at the same time show that the bacteriophage requires the *p*-aminobenzoic acid-containing intermediates which probably catalyse the synthesis of methionine, xanthine, serine and valine.

It is thought that the submitted facts are a possible approach to virus chemotherapy as it is shown that interference with host-cell metabolism can inhibit virus propagation without unduly affecting the host cell.

SUMMARY.

It is shown that high concentrations of sulphanilamide completely inhibit the intracellular multiplication of the bacteriophages T_2, T_4 and T_6 on *Escherichia coli* B, if the interference of sulphanilamide with bacterial growth is antagonized with four non-competitive sulphanilamide antagonists. This effect is due to an interference with bacterial metabolism and might be a possible approach to virus chemotherapy.

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STUDY OF A CASE OF PURPURA ASSOCIATED WITH BONE CHANGES AND FORMATION OF A GEL IN THE SERUM ON COOLING.

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WALDENSTRÖM (1943) described the cases of three women who had long-standing purpura associated with a high concentration of globulin in the serum. Lerner and Watson (1947) have described the case of a man who was abnormally sensitive to cold for 9 years and had recurrent purpura for 7 years before death ; when this patient's serum was cooled a protein precipitated spontaneously. They mention five reports of the association of purpura or recurrent haemorrhage with a high concentration of serum globulin ; in two of these (Wintrobe and Buell, 1933 ; Flemberg and Lehman, 1944), proteins precipitated spontaneously from the serum.

In a case diagnosed as " aleukaemic plasma-cell leukaemia," described by Hansen and Faber (1947) the patient was abnormally sensitive to cold and frequently bled from the nose, but had no subcutaneous haemorrhages ; when the patient's serum was cooled, protein separated in the form of granules. In one of the cases which Waldenström (1944) classed as " incipient myelomatosis," the serum formed a gel on cooling ; this patient bled repeatedly from the nose, but did not have purpura. More recently, Hill, Dunlop and Mulligan (1949) described the case of a patient with myelomatosis who was sensitive to cold ; no mention is made of the appearance of purpura. This patient's serum solidified on cooling.

Lerner and Watson (1947) suggest that the group of serum proteins that have the common property of precipitating or forming a gel on cooling, should be

called " cryoglobulins." To the list of examples given by them may be added two described by Holmberg and Grönvall (1942) and Packalén (1939) and another one described by Shapiro and Ross (1946) of proteins which crystallized from the serum ; the first two patients had myelomatosis, and the third had periarteritis nodosa. The chemical and physical properties of the abnormal protein found in 5 cases have been studied by von Bonsdorff, Groth and Packalén (1938), Holmberg and Grönvall (1942), Shapiro, Ross and Moore (1943), Lerner and Greenberg (1946), and Hill, Dunlop and Mulligan (1949).

The patient discussed in this article had purpura, and his serum contained an abnormal protein which formed a gel on cooling.

CLINICAL HISTORY.

January, 1944.

At this time S. S—, aged 57, a farm labourer, first complained of purpuric spots and swellings on, and pains in the bones of his legs, and occasional small haemoptyses. Later in the year his vision became misty ; this cleared in a few weeks and was followed by a sudden enlargement of his salivary glands on both sides. These swellings also subsided.

July, 1945.

He first came under our notice when he was admitted to Haymeads Hospital, Bishop's Stortford. Then, in addition to heavily blood-streaked sputum and purpura confined to the legs below the knees, he complained of pains in the chest, long bones and back of neck ; drumming in the ears, slight deafness, and loss of weight. He said haemoptysis only occurred when his purpura was bad. He had clubbing of the fingers and a slight diffuse swelling of the soft tissues in the upper part of the left forearm which he said resembled the swellings which came and went on his legs. There was slight ptosis of his left eye and he was anaemic. The lymph glands were not enlarged, and the liver and spleen were not palpable.

X-ray of bones.—Increased density and coarse trabeculation of the whole skeleton, with marked thickening of the cortex in the long bones. The patient was discharged.

October, 1947.

His condition was similar to that of 1945. He had blood-streaked sputum and purpura confined to his legs. He had cycled 10 miles to hospital, and said that this had made his purpura worse. Exercise always had this effect, but he had not noticed any relationship to cold. The chest was dull at the right base, apparently owing to a pleural effusion. The left pupil was larger than the right, with normal reactions. The skull circumference was 22¾ in. Though large, he said he had not noticed his head getting larger. There was no bowing of the long bones. The blood pressure showed a large pulse pressure 142/75. Pulse rate 108. Hess test positive on arm. The temperature showed occasional rises to 101°. One cervical lymph gland was palpable. The liver and spleen could not be felt.

X-ray of bones.—The appearances were the same as in 1945. There appeared to be a dramatic response to oral ascorbic acid in that haemoptysis ceased and the positive Hess test became much weaker. As he had been in bed two weeks before

ascorbic acid was given the change did not appear to be due to rest alone. Unfortunately no analyses for ascorbic acid had been made previous to treatment. His diet had been normal. The response to continued ascorbic acid was not fully maintained.

Patient was discharged after 4 weeks in hospital.

March, 1949.

He now has purpura not only on his legs, but on his hands and complains of pains in limbs and head, but is able to carry out light duties at home.

TABLE I.—*Laboratory Examinations.*

	July, 1945.	Oct., 1947.	March, 1949.
Haemoglobin, per cent (Haldane)	60	68	
Red blood cells, million per mm.	3·1	3·5	
Colour index	0·97	0·97	
White cells (per mm.):	9200	9100	
Polymorph	73 per cent	78 per cent	
Lymphos.	23 ,,	18 ,,	
Monos.	2 ,,	3 ,,	
Eosinos.	2 ,,	1 ,,	
Platelets, per mm.	279,000	Plentiful	
Abnormal cells	0	0	
Sternal marrow	Normal	Normal	
Bleeding time	,,	,,	
Clotting time	,,	,,	
Clot retraction time	,,	,,	
Prothrombin time	,,	,,	
E.S.R. (Westergren): mm./hour	128	140	
Serum protein, g./100 ml.	7·6	7·5	
,, albumin, g./100 ml.	3·5	3·6	
,, globulin, g./100 ml.	4·1	3·9	
Alkaline phosphatase units (Haslewood & Delory)	12 (normal, 5–10)	17	22
Acid phosphatase units (Haslewood & Delory)	1·5 (normal 0·5–2)	1·3	
Inorganic phosphate, mg./100 ml.	3·7	3	
Calcium, mg./100 ml.	..	10·9	
Blood urea, mg./100 ml.	..	55	
Cholesterol, mg./100 ml.	95	..	

No Bence-Jones protein or other abnormal constituents were found in the urine. No tubercle bacilli were found in the sputum. The Wassermann reaction was negative.

Diagnosis.

This would seem to rest between Paget's disease and a reticulosis. The X-ray appearances favour Paget's disease. The alkaline phosphatase, however, which is usually very high in this disease, is only slightly raised. The history of the eye trouble and enlargement of the salivary glands is suggestive of the uveoparotid syndrome which occurs in the reticulo-endotheliosis known as sarcoidosis.

Effect of cooling on serum and plasma.

It was observed that both his serum and plasma became cloudy and solid at room temperature and clear and fluid again when warmed. This reversible reaction could be repeated almost indefinitely. More exact observations on 1 c.c. of serum contained in a small thin test tube 3 in. $\times \frac{3}{8}$ in. suspended in a glass water-bath constantly stirred, which was slowly warmed or cooled by stages, gave the following results:

From cloudy and solid to clear and fluid.

Temperature.	Condition of Serum
23° C. . .	Cloudy, solid gel.
24° C. . .	Slightly less cloudy. Viscous.
25° C. . .	Definitely less cloudy. Fluid. Kept at this temperature for ½ hour the degree of cloudiness remained constant.
27° C. . .	Quite clear. Fluid.

From clear and fluid to cloudy and solid.—The same results were obtained in the reverse direction on cooling by stages from 27° C. to 23° C.

ISOLATION OF THE GEL PROTEIN.

The diagram Table II indicates the procedure used for the isolation of the gel protein. "Sn3" was very viscous at 0° but did not form a gel. On adding HCl until a pH of about 6·2 (indicator paper) was reached, the solution formed a gel. On further addition of HCl a white precipitate separated which occupied most of the total volume. On high speed centrifugation this precipitate became translucent, with little (further) reduction in volume. This precipitation with acid, therefore, hardly improved the purity of the preparation.

TABLE II.—*Isolation of the Gel Protein from Serum I.*

(S) serum 3·7 ml. centrifuged

- gel 1·7 ml.; +2 ml. 0·85 per cent NaCl, cooled, centrif. at 1°
  - (P1) gel 1 ml.; +2 ml. 0·85 per cent NaCl cooled, centrif. at 1°
    - (P2) gel 1 ml.; +1 ml. 0·85 per cent NaCl, cooled to 0°; did not form gel.
    - (Sn2) supernatant 2 ml.
  - (Sn1) supernatant 2·5 ml., dialysis first against N/10^5 HCl, then against N/10^6 HCl, centrif.
    - trace of white precipitate, discarded
    - (Sn1′) supernatant, contained 65 per cent albumin.
- (Sn0) supernatant 2 ml.

(P2) and (Sn2) combined, evaporated to dryness *in vacuo*, dissolved in about 0·7 ml. H_2O and dialysed against N/10^6 HCl, centrif.

- (P3) precipitate, 0·1–0·2 ml.
- (Sn3) supernatant evaporated to dryness *in vacuo*, dissolved in 0·3 ml. H_2O + N/10^4 HCl till pH about 5·5–5·8 (methyl red); white precipitate, centrif. at high speed
  - (P4) precipitate, about 0·35 ml.
  - (Sn4) small amount of supernatant, discarded.

Electrophoresis.

The experiments were made in the Tiselius apparatus equipped with the cylindrical lens schlieren optical system. Phosphate buffer of pH 8 and ionic strength 0·1 was used. The results summarized in Table III and Fig. 1 indicate that the gel protein is represented in the pattern by the large peak (x) which moved with the mobility of the β-globulins. This component is seen in the patterns of sera obtained on two occasions (Fig. 1*a* and *b*). The composition of these sera was similar and the patterns did not differ except for the position of the anomaly in the descending limb which occurred near the α_2-boundary in one case. In a portion of Serum I which had liquified on keeping 6 weeks in the cold, the proportions of the components were unchanged.

TABLE III.—*Analyses of Electrophoretic Patterns of Sera forming Gels and of Fractions.*

(Components as per cent of total refractive area.)

	A+α_1.	α_2.	β+gel protein.	γ.	Total concentration of protein in solution electrolysed; g./100 ml.	Time of electrophoresis at or corrected to 9·3 V/cm. (Minutes.)
Serum 1	44·0	5·4	40·5	10·1	0·86	82
„ 2	43·8	7·2	38·4	10·6	0·85	80
„ 1 liquified on keeping	43·5	8·6	38·1	9·8	0·86	Not shown in Fig. 1.
Sn0	61·8	11·6	11·9	14·7	*ca.* 0·8	82
Sn1′	*ca.* 65		*ca.* 35		*ca.* 0·2	Not shown in Fig. 1.
P3	..		95–100*		*ca.* 0·7	113 (run at 6V/cm.)
P4	5		95		*ca.* 0·3	89

* One peak only.

The preparation " Sn0 " (Fig. 1*c*) also showed the anomaly at the α_2-peak. Preparation " Sn1′ " contained 65 per cent albumin, and other material was distributed diffusely over the region of the globulins. Preparation " P3 " showed one well defined component and there was no indication of impurities, but an additional peak of 5 per cent or less of the total area would not have been distinguishable. In preparation " P4," which contained the bulk of the gel protein originally present, one peak made up 95 per cent of the total area and migrated with the mobility of β-globulin. The only impurity discernible was albumin.

Solubility.

The isolated gel proteins were soluble in water and in NaCl solution ; Lerner and Greenberg (1946) also found that their cryoglobulin became soluble in saline after repeated precipitation. No precipitate or gel was formed on addition of a small amount of " Sn0 " of human serum albumin or of $CaCl_2$ to a concentrated solution of " P4." On the other hand the original gel protein was only very slightly soluble in serum at room temperature, as shown by the electrophoretic pattern of " Sn0 " (Fig. 1*c*) in which there is only a small β-globulin peak. Some component may, therefore, have been removed on dialysis or the protein may have been structurally altered during the preparation.

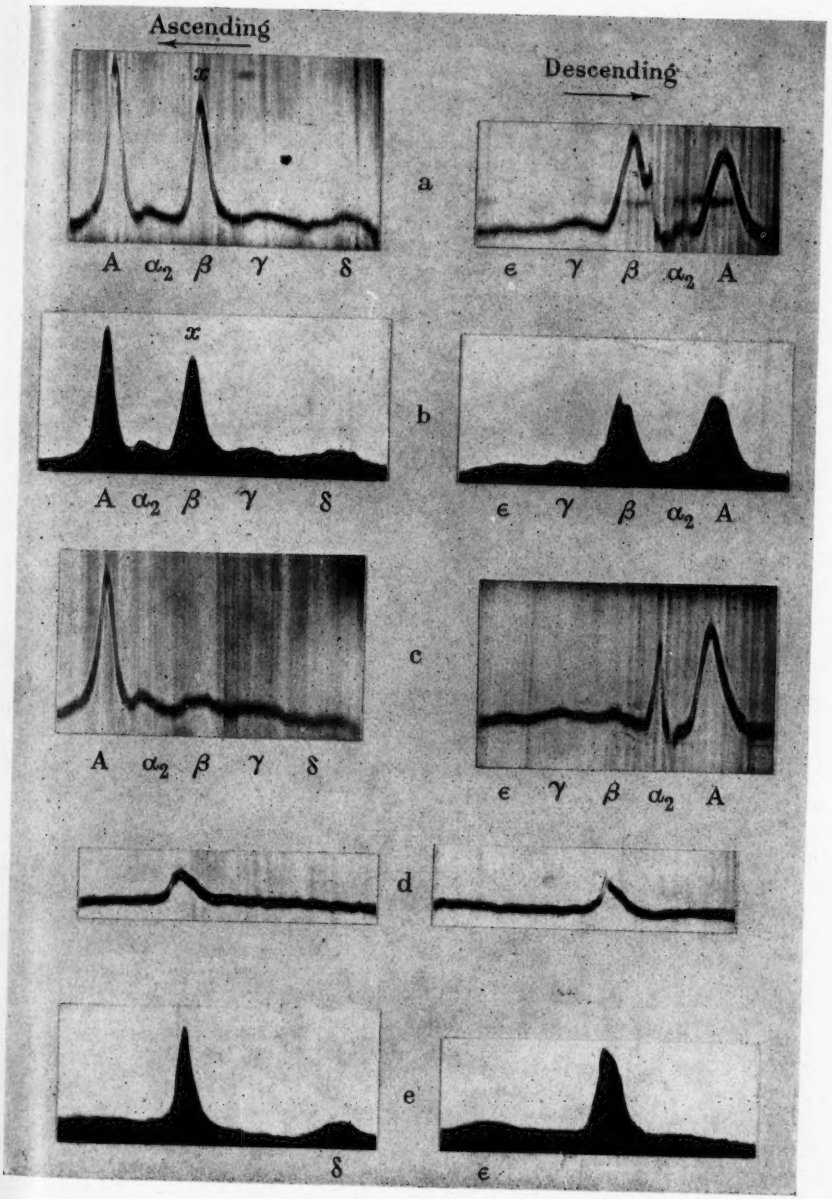

FIG. 1.—(*a*) Serum 1, (*b*) serum 2, (*c*) Sn0, (*d*) P3, (*e*) P4.

Chemical analysis of the isolated protein.

All the chemical tests were made, after the electrophoresis experiment, on portions of the solution of " P4 " in phosphate buffer.

(*a*) Cholesterol, by photoelectric measurement of the colour developed in the Liebermann-Burchardt reaction, under 0·5 per cent.

(*b*) Lipid phosphorus, none found (i.e. under 0·03 per cent) by a photoelectric modification of the method of Fiske-Subarow.

(*c*) Total lipids 7 per cent or less. The sample may have contained normal β-globulin, as this would not be detected by electrophoresis. Since normal β-globulin may contain 30 per cent of lipids the analysis of lipids was not pursued further.

(*d*) Carbohydrate 5·9, 5·7 per cent (as galactose-mannose) estimated photoelectrically by the carbazole method (Holzman, MacAllister and Niemann, 1947).

Sedimentation constant.

Preparation P4 (see Table II) was examined in the ultracentrifuge by Dr. Kekwick, at the Lister Institute. The sedimentation constant, S_{20}, was found to be $17{\cdot}2 \times 10^{-13}$, assuming a specific volume of 0·75. The molecular weight works out to between 400,000 and 1,000,000 if the frictional ratio is assumed to be between 1 and 2·5.

Absorption spectrum.

Preparation P4 was examined in phosphate buffer pH 8·0 and in $n/10$ NaOH with a Hilger Spekker photometer and Medium Quartz spectrograph using an M.S.C. low voltage hydrogen are as source. Unfortunately, owing to an error, no nitrogen estimation was made on the sample so that no specific extinction coefficient can be given for the protein. It was, however, possible to analyse the absorption curve for the proportion of tyrosine to tryptophane as described by Holiday (1936) and Holiday and Ogston (1938).

A molar ratio of tyrosine : tryptophane of 1·63 : 1 was found. This represents a higher proportion of tryptophane than was found in β- or γ-globulins, or fibrinogen by Brand, Kassell and Saidel (1944), who found ratios of 3·0, 2·66 and 1·97 respectively. Experience has shown that the spectrographic estimation tends to give too high an estimate of tyrosine as compared with chemical methods. This would have the effect of making the figure of 1·63 too high, and would therefore indicate an even greater difference between the gel-protein and other serum components.

IMMUNOLOGY.

The reactions of two preparations " P3 " and " P4 " were tested with two kinds of antisera :

(A) *Antiserum to γ-globulin.*

The γ-globulin was prepared by Kendall's (1937, 1938) method. The proteins obtained in this way contained less than 6 per cent of globulin other than γ-globulin. Kendall called the globulin fraction made in this way α-globulin, but suggested (1937) that it was the same as the γ-globulin of Tiselius. Incidentally,

antisera prepared in this way have proved very satisfactory for the Coombs-Mourant reaction (Coombs, Mourant and Race, 1945). They can be used, without preliminary clearing, in dilutions of over 1 in 1000. Rabbits were immunized by intramuscular injections of antigen precipitated with alum (Proom, 1943), at intervals of a fortnight. The antigen was precipitated freshly for each injection instead of being precipitated in bulk and stored in the precipitated state, as recommended by Proom. Almost all the antibodies in this serum were antibodies to γ-globulin, for the maximum precipitate obtained with normal human serum was only about 5 per cent more than that obtained with γ-globulin, and after clearing with γ-globulin the antiserum formed no precipitate with normal human serum.

(B) *Antiserum made by immunizing a rabbit with the floccules formed in the thymol-flocculation reaction* (*Maclagan*, 1944).

The floccules were formed by the technique described by Cohen and Thompson (1947); they were washed three times with cold thymol-buffer, suspended in saline and precipitated with alum (Proom, 1943). About 80 per cent of the antibodies in this antiserum was precipitated by γ-globulin. The reactions of these antisera are discussed further in a paper on the immunological aspects of the thymol turbidity reaction (Marrack, Johns and Hoch, 1950).

The amounts of precipitate formed when varying amounts of antigen were added to constant amounts of antiserum were measured. Into each of a series of centrifuge tubes 0·5 ml. of antiserum was added. About $\frac{1}{4}$ of the optimum amount of antigen was then added to the first tube and increasing amounts up to about three times the optimum added to the other tubes. The volumes were made up to 2 ml. with saline. The mixtures were left at room temperature until floccules formed and were then kept at 4° for two days; the precipitates were centrifuged down, washed three times with cold saline, and the protein in the precipitate was measured by a modification of the method of Folin and Ciocalteu (Heidelberger and MacPherson, 1943, 1944).

The method of Oudin (1947) was used to detect the presence of more than one antigenic protein. In this method distinct antigens are detected by the formation of separate bands in a column of agar, containing antiserum. Antisera were sterilized by passage through a Seitz filter and solutions of gel protein by filtration through collodion membranes.

(A) When gel protein, preparation "P3," was added to an antiserum to γ-globulin ("Gray" 8/1/48) floccules formed very much more slowly than when γ-globulin from normal serum was added to this antiserum. The maximum precipitates formed with gel-protein was only about half that formed with γ-globulin. When the antibody to γ-globulin was completely precipitated by addition of γ-globulin the supernatant fluid no longer formed a precipitate on addition of preparation "P3."

This type of reaction resembles a cross-reaction of an antiserum with a protein resembling, but not identical with the homologous antigen; for example, the reaction of antiserum to hen-egg albumin with duck-egg albumin (Osler and Heidelberger, 1948).

By the method of Oudin (1946) normal human serum formed two bands with the antiserum "A" to γ-globulin. One of these was, presumably, due to the formation of a precipitate by γ-globulin with its antibody and the other to

the formation of a precipitate by a second constituent of human serum with a second antibody in the serum. Gel protein preparation " P3 " formed one band only, and therefore contained but one antigen that would react with the antiserum. Preparation " P4 " formed a sharp band and a haze and therefore contained some of the second constituent of human serum that reacts with the second antibody in the antiserum.

(B) Flocculation was slow when either preparation " P3 " or " P4 " was added to antiserum to thymol-floccules. The maximum amount of precipitate formed with preparation " P3 " was only about half the maximum formed with normal serum and about two-thirds of that formed with γ-globulin. The curve obtained with preparation " P4 " fitted closely to that given by normal serum.

All the antibodies to globulin fractions, other than γ-globulin were removed from this antiserum by repeated additions of small amounts of a solution of α- and β-globulin, which contained little γ-globulin. The cleared antiserum still formed a precipitate with γ-globulin, and formed a precipitate slowly with preparation " P3." After precipitation of the antibodies to γ-globulin by addition of γ-globulin, the supernatant fluid no longer found a precipitate with preparation " P3."

By the method of Oudin normal human serum formed two bands with antiserum to floccules. Gel protein preparation " P4 " formed a sharp band and a haze.

Preparation " P3 " seems to have one constituent, which was not identical with γ-globulin, but cross-reacted with antiserum to γ-globulin. It may be inferred that the gel protein resembles γ-globulin more closely than it resembles the other fractions of serum globulin, but differs in structure or composition. Preparation " P4 " contained more than one protein.

DISCUSSION.

Our patient differed from most of those described in that he was not abnormally sensitive to cold. It has been suggested that the sensitiveness to cold is due to impairment of circulation owing to increased viscosity of the plasma or actual formation of precipitates on cooling. In the case of Flemberg and Lehman (1944) the protein precipitated at 33°, and in that of Hansen and Faber (1947), the abnormal protein was very little soluble at 30°. In our case the serum or plasma was quite fluid at 27°. It is possible that the peripheral circulation of our patient was less liable to impairment by cold because the temperature at which the abnormal protein separated out was lower than in other cases.

Lerner and Watson (1947) quote 9 cases in which protein separated from the patient's serum on cooling ; 5 of these patients had myelomatosis. The patients of Flemberg and Lehman (1944) and of Hill *et al.* (1949) also had myelomatosis. In the case of Hensen and Faber (1947) the medullary spaces of the bones were filled with a dense tissue very rich in plasma cells and there were diffuse plasma cell infiltrations in the splenic pulp. Lerner and Watson do not mention any examination of the bones of their patients either by X-ray or post-mortem. In a case described by Shapiro, Ross and Moore (1943), in which myelomatosis was associated with purpura, a peculiar viscous protein was found in the serum. The X-ray pictures of the bones of our case did not resemble those usually found with myelomatosis and myeloma cells were not found in the sternal marrow on two occasions. Also, the patient has lived for 5 years since he was first seen.

Patients with multiple myeloma rarely live so long, although recent studies have shown that their lives are not always as short as had originally been supposed (Lichtenstein and Jaffe, 1947).

The abnormal protein in the serum of our patient differed from those described by Von Bonsdorff, Groth and Packalén (1938), Holmberg and Grönvall (1942), and by Lerner and Greenberg (1946), in that it formed a gel rather than a precipitate on cooling. Also its apparent molecular weight was higher than that of these three and of the gelatinous protein of Shapiro, Ross and Moore (1943). Its sedimentation constant was slightly less than that of the abnormal protein in the serum of two of Waldenström's (1944) patients, one of which formed a gel on cooling. It is possible that the molecular weight was in the neighbourhood of 1,000,000 and that the molecule was very elongated and formed a gel as fibrinogen does. Our protein, like that of Hill *et al.* (1949), contained carbohydrate, while that of Lerner and Greenberg (1946) did not; the protein of Hill *et al.* as separated contained a considerable amount of cholesterol, whereas ours did not. Lerner and Greenberg found that their protein resembled γ-globulin in absorption spectrum and nitrogen content. They did not, however, study the electrophoresis of this protein or of the whole serum at a pH near 8. The viscous protein of Shapiro, Ross and Moore had the mobility (pH 7·8, $\mu = 0{\cdot}05$) of γ-globulin. Although the mobility of our protein at pH 8 was the same as that of the normal β-globulin of serum it did not contain the large amount of phosphatide and cholesterol usually found in β-globulin. However, the lipo-protein is only one member of the group of proteins that are included in β-globulin; it is possible that the amount of a member of the normal β-globulin group, that does not contain a large amount of lipid, might be increased.

Immunologically the abnormal protein cross-reacted with γ-globulin and appeared to differ from β-globulin.

SUMMARY.

The case is described of a patient who suffered from purpura with changes in the bones that did not resemble those of myelomatosis.

The patient's serum formed a gel on cooling.

The patient's serum contained an abnormally large amount of a globulin with the mobility at pH 8, of β-globulin. When the protein that formed a gel on cooling was removed this component was much reduced.

The protein that formed the gel on cooling was isolated. Its composition and physical properties were studied. The isolated protein cross-reacted with antiserum to normal γ-globulin.

Our thanks are due to Mr. L. Bishop for sterilizing the solutions of gel protein.

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THE METABOLISM OF CELL DIVISION.

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ATTEMPTS have been made to demonstrate increased cellular respiration during mitosis by measuring the variations in oxygen consumption of segmenting ova; and in amphibia, Brachet (1944), Stefanelli (1937) and Zeuthen (1946) have recorded increases in oxygen consumption associated with cell division. On the other hand there are experimental results indicating that growth involving cell division can occur without the use of oxygen. Laser (1933) has recorded such a finding in fibroblasts cultured *in vitro*, and Brachet (1934) found that frog ova segmented and developed as far as the blastula stage when oxygen consumption was reduced to 10 per cent normal by cyanide.

It is therefore necessary to consider alternative sources of energy in cell division, and there is evidence to suggest that such energy may be derived from the breakdown of carbohydrate by aerobic glycolysis. Pomerat and Willmer (1939) reach this conclusion from the effect of metabolic inhibitors on the growth of tissue cultures. They conclude that the actual process concerned is the breakdown of glucose without phosphorylation, identifying it with the process of carbohydrate metabolism described by Needham and Lehmann (1937*a*, 1937*b*) in the chicken embryo. It has to be mentioned, however, that Dorfman (1943) does not consider that there is adequate evidence for such a form of carbohydrate metabolism.

Further, the process described by Needham and Lehmann does not occur aerobically, and the work of Pomerat and Willmer suffers from the limitation that carbohydrate metabolism was not quantitatively measured, their conclusions being based on the similarity of the concentrations inhibiting cell growth and the concentrations inhibiting carbohydrate breakdown in other circumstances.

Thus in the case of both respiration and glycolysis there is suggestive but not conclusive evidence of a direct association with cell division. This paper records experiments using embryonic tissue in which the developmental variations in all three processes have been measured in an attempt to find relationships between them.

MATERIAL.

In all observations the midbrain of the chicken embryo has been used, and the developmental period covered was the third to eighth days of incubation. Since at a given time of incubation, the developmental stage reached varied slightly, this was identified by the diameter of the eye, and results have been related to this measurement. All observations were made on the dorsal half of the midbrain and on an area lying adjacent to the midline. When, as a result of development, a prominent median sulcus developed, this was avoided in selecting the area for investigation.

METHODS.

1. *The estimation of the number of dividing cells.*

For this purpose the dividing cells were counted in serial sections, and the results expressed as the number of dividing cells per c.mm. tissue. The accuracy of this measurement thus depends on the accuracy with which sections were cut at a required thickness. The possibility of error from this source was investigated by comparing the results from different sets of sections cut from the same block. Since no significant difference was found it was concluded that the microtome delivered sections of a constant thickness. No investigations were made as to whether the thickness was exactly as the microtome setting, for errors from this source would equally affect all measurements and therefore not be important when relative values for dividing cells were considered. All sections were cut with the same microtome at a setting of 6μ.

2. *The measurement of respiration.*

Embryos were removed from the egg into the following medium :

NaCl . .	0·9 g.
Kcl . .	0·02 ,,
$MgCl_2$ . .	0·02 ,,
$CaCl_2$ . .	0·02 ,,
Glucose .	0·10 ,,
Water . .	100 c.c.

to which 10 c.c. of M/15 phosphate buffer was added to produce pH 7·4.

After stripping the midbrain of its coverings with watchmaker's forceps, a piece of tissue 0·1 to 0·4 c.mm. in volume was removed with iridectomy scissors and used for measuring the respiratory rate. The volume was measured by compressing the tissue beneath the coverslip of a cell-counting chamber and deriving the volume from the area covered and the depth of the chamber. The depth of the chamber was suited to the thickness of the tissue so that it was compressed evenly, but not excessively. Two depths were used, firstly 0·1 mm. in the standard haemocytometer, and secondly a depth of 0·06 mm. obtained by placing a piece of coverslip in a chamber of 0·2 mm. depth. These manoeuvres were carried out beneath the surface of the medium contained in a Petri dish.

The measurement of the respiratory rate was carried out by a modification of the Cartesian diver micromanometer (O'Connor, 1948, 1949). The divers used were of the proportions indicated in Fig. 1, and of the following approximate dimensions :

Neck length	15 mm.
Internal neck diameter . . .	1·2 mm.
Total capacity	50 c.mm.

The filling of the divers followed the technique of Boell, Koch and Needham (1939). The bulb contained the tissue suspended in 10 to 15 c.mm. of medium (Fig. 1*a*), the atmosphere was oxygen, and in the neck there were seals of M/6 $Ba(OH)_2$, HCl, liquid paraffin and flotation fluid (O'Connor, 1948). With this arrangement the oxygen consumed could be directly measured since the barium

hydroxide absorbed the carbon dioxide produced. This was subsequently measured by adding the acid and the alkali to the bulb, where excess of acid released the absorbed carbon dioxide. The respiratory quotient was thus calculated, and, at all developmental stages, did not differ significantly from 1·0.

In these measurements, as in the case of aerobic glycolysis, it was necessary to calculate the factors connecting change in equilibrium (flotation) pressure and the volume of gas change in the diver. These were calculated according to the formulae given by Boell, Needham and Rogers (1939). One point should, however, be noted. These authors introduce a factor to allow for unequal movement of fluid in the two arms of their manometer. Such a factor is not

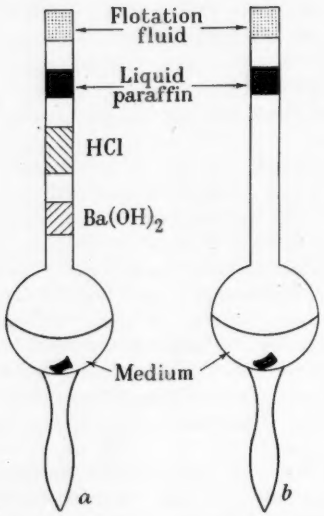

FIG. 1.—Arrangement of divers in the micromanometric technique. (*a*) For respiration and respiratory quotient. (*b*) For aerobic glycolysis.

needed in the present modification, but the point is mentioned because Humphrey and Humphrey (1948) state that the factor is incorrectly calculated.

The rates of respiration at various stages are recorded as c.mm. O_2/c.mm. tissue/hour.

3. *The measurement of aerobic glycolysis.*

Needham, Rogers and Shen Shih-Chang (1939) have reproduced in the Cartesian diver technique the double cup method of Warburg in order to measure aerobic glycolysis. However, attempts to follow this technique produced unsatisfactory results. Aerobic glycolysis was therefore calculated from the total gas change in the diver arrangement shown in Fig. 1*b*. The bulb contained 0·1 to 0·4 c.mm. tissue suspended in the following medium :

NaCl	. .	0·9 g.
KCl	. .	0·02 ,,
CaCl	. .	0·02 ,,
$MgCl_2$	. .	0·02 ,,
Glucose	.	0·10 ,,
$NaHCO_3$	.	0·20 ,,
Water	. .	100 c.c.

the diver being filled with a gas mixture of 5 per cent carbon dioxide and 95 per cent oxygen. The neck contained seals of paraffin oil and flotation fluid.

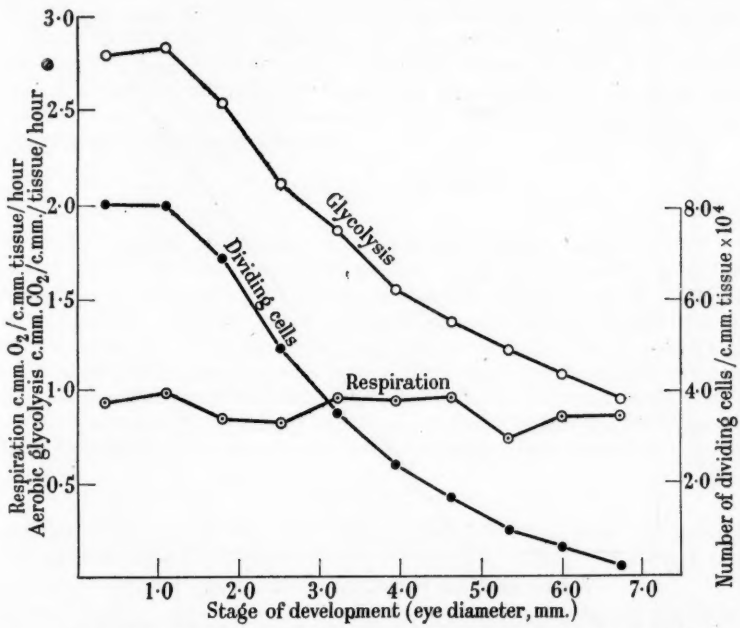

FIG. 2.—Variations during development of respiration, aerobic glycolysis and dividing cells.

With such a diver arrangement the total gas change is made up of three components: oxygen up-take, respiratory carbon dioxide, and carbon dioxide released from the bicarbonate by acid formation. The assumption was made that values for the first two were the same as the values found in the phosphate buffered medium used to measure oxygen consumption directly. As Dixon (1943) has pointed out, this assumption is not necessarily justified, but such results as could be obtained by the double cup modification showed that any difference was not great enough to invalidate the results obtained in view of the following considerations. Firstly, the pressure changes due to oxygen consumption and respiratory carbon dioxide are in opposite directions and so will to a large extent "cancel out." Secondly, as will be recorded below, at all stages

the rate of respiration and evolution of respiratory carbon dioxide are the same, so that any errors introduced by the assumption made will be similar at all stages and hence become of lessened significance when relative rates are considered.

Rates of aerobic glycolysis are recorded as c.mm. CO_2/c.mm. tissue/hour.

RESULTS.

In order to record results, the developmental period, 3rd to 8th day of incubation, has been divided into ten intervals each represented by an increase of 0·7 mm. in the diameter of the eye. Values obtained for dividing cells, respiration and aerobic glycolysis were grouped into these ten intervals, and

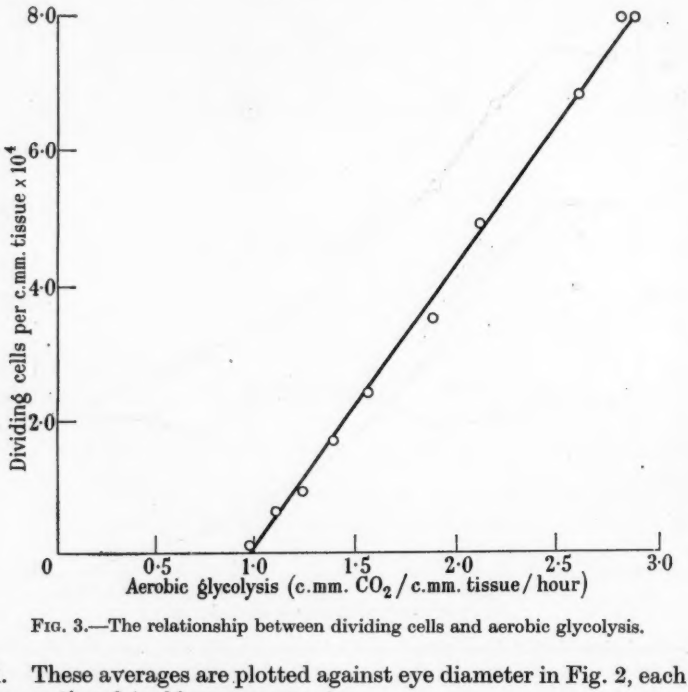

FIG. 3.—The relationship between dividing cells and aerobic glycolysis.

averaged. These averages are plotted against eye diameter in Fig. 2, each plotted point representing 6 to 10 measurements.

The respiratory rate, it will be seen, undergoes no significant variation from a rate of 0·8 c.mm. O_2 c.mm. tissue/hour in the developmental period considered. On the other hand both the number of dividing cells and the rate of aerobic glycolysis undergo a decrease in this period, with trends that are similar.

DISCUSSION.

From Fig. 2 it will be seen that although the number of dividing cells approximates to zero in the period considered there is no measurable decrease in the rate of oxygen consumption. However, inaccuracies in the measurement of the respiratory rate make it possible that the respiratory rate at an eye diameter

of more than 6·0 mm. is less than the rate at 1·0 mm. by about 10 per cent. At an eye diameter of 1·0 mm. total cell counts indicate that the proportion of dividing cells is between 5 to 10 per cent, while at eye diameter of over 6·0 mm. this proportion can be, in the present consideration, taken as zero. Hence the results of Fig. 2 do not entirely eliminate the possibility that a loss of 5 to 10 per cent dividing cells is associated with a decrease of 10 per cent in respiration allowing of the possibility that dividing cells use more oxygen than non-dividing cells. This is, however, putting the extreme case, and the relationship between respiration and number of dividing cells can reasonably be considered consistent with the view of Brachet (1944) that, in cell division, if there is a small increase of oxygen consumption the metabolic cause of this is not essential to division.

In Fig. 2 the lines for dividing cells and aerobic glycolysis indicate that the changes they undergo in development are similar. This similarity is further illustrated by the linear relationship obtained when in Fig. 3 corresponding values for the rates of aerobic glycolysis and the number of dividing cells per c.mm. are plotted against one another. The relationship between the two measurements may thus be expressed :

$$M = aG - b$$

where M is the number of dividing cells for c.mm. tissue, G the rate of aerobic glycolysis expressed in the terms of the present experiments, and a, b constants.

Although such a relationship is not necessarily causal, such a possibility is worth consideration. It only requires a small amount of extrapolation in Fig. 3 to show that when the number of dividing cells is zero the rate of aerobic glycolysis is 0·9 c.mm. CO_2/c.mm. tissue/hour. This may indicate that with regard to cell division aerobic glycolysis has a threshold value or alternatively that there is a fraction of aerobic glycolysis of which cell division is independent, and which might then be associated with cellular activities apart from division.

SUMMARY.

In the mid-brain of the chicken embryo the following have been measured at the different stages of development : number of dividing cells, rate of respiration, and rate of aerobic glycolysis.

Between the third and eighth days of incubation the number of dividing cells decreases from $8·0 \times 10^4$ per c.mm. to $0·04 \times 10^4$ per c.mm.

During this period there is no measurable alteration in the rate of respiration ; but the rate of aerobic glycolysis decreases from 2·8 c.mm. CO_2/c.mm. tissue/hour to 0·9 c.mm. CO_2/c.mm. tissue/hour.

The variations in the number of dividing cells and the rate of aerobic glycolysis are such that a linear relationship can be established between the two.

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THE ESTIMATION OF MITOTIC INHIBITION.

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So many authors have measured mitotic inhibition by the use of tissue cultures that this can be regarded as the standard *in vitro* method. However, in a previous publication (O'Connor, 1949) the effect of acridine compounds on dividing cells was estimated by exposing fragments of embryonic chicken brain to various concentrations of the compounds in a saline solution. Adequate concentrations of the compounds produced a pycnotic fusion of the chromatin material in dividing cells which could be detected by squash preparations. Although this represented a simple method for demonstrating action by mitotic inhibitors, it could not be certain that a comparison with tissue culture methods was valid owing to the differing conditions. Further, the manner of estimating the final result was different, since tissue culture estimations involve a quantitative examination of dividing cells or of total growth. A number of compounds have been tested using embryonic brain tissue, and the results compared with those recorded by other authors using tissue cultures. Since the technique with embryonic tissue has been modified, technical details are given.

MATERIAL AND METHODS.

Throughout the following medium was used:

NaCl . .	0·90 g.
KCl . .	0·02 ,,
$MgCl_2$ . .	0·02 ,,
$CaCl_2$ . .	0·02 ,,
Glucose .	0·10 ,,
Water . .	100 c.c.

to which 10 c.c. of M/15 phosphate buffer was added to produce pH 7·4.

Six-day chicken embryos were removed from the shell into a Petri dish containing the above medium and the dorsal half of the midbrain removed with iridectomy scissors. The coverings of the midbrain were taken off with watchmaker's forceps, and the brain tissue cut into fragments 1 to 2 mm. square. The stage of development used was of some importance, since at the sixth day there are sufficient dividing cells to facilitate observation, and at this stage the best spread of cells was obtained when squash preparations were made.

Serial dilutions of the compounds to be tested were made in 2 to 3 c.c. of medium contained in test tubes 8 cm. × 1 cm. The brain fragments were then transferred to these tubes with a minimum amount of fluid, so that the resulting

alteration in concentration could be neglected. The fluid in the tubes was then reduced to a layer 1 to 2 mm. in depth. The air in the tubes was replaced with oxygen and the tubes corked. The tubes were then incubated at 37° C. for 90 minutes.

At the end of this time the fragments of tissue were fixed and stained by the aceto-lacmoid method of La Cour (Darlington and La Cour, 1942). Both fixative and stain were applied to the tissue in watch glasses, the fixative for 15 minutes, and the stain for 10 minutes. During the staining period the watch glasses containing tissue and stain were warmed three times over the pilot flame of a Bunsen burner until vapour rose. Squash preparations were then made, ringed with vaseline, and examined forthwith. The appearances, in such preparations, of normal mitoses and the change produced by an effective concentration of inhibitor have been previously recorded (O'Connor, 1949, Fig. 1, 2). The change consists of a fusion of chromatin material in dividing cells into a pycnotic mass and occurred with all the compounds tested. The concentration producing this change will be referred to as the effective mitotic concentration (E.M.C.). At half this concentration changes were slight or absent.

RESULTS.

The E.M.C. values of the compounds tested are recorded in Table I, together with the inhibiting concentrations found by other authors using tissue culture methods. These authors have all used cultures of fibroblasts, but the method of measurement varied and is indicated in Table I.

TABLE I.—*The Inhibition of Dividing Cells in Embryonic Tissue and in Tissue Culture.*

Compounds.	E.M.C. mg./100 c.c.	Inhibition in tissue culture mg./100 c.c.	Authors.	Method of estimation.	Tissue culture/ E.M.C.
5 amino-acridine hydrochloride	0·8	0·4	Lasnitzki and Wilkinson (1948)	Number of dividing cells	0·5
5 amino-10-methyl acridine bromide	0·7	0·4	Do.	Do.	0·6
5 amino-1-phenyl-10-methyl acridine bromide	1·8	3·0	,,	,,	1·7
Sodium azide	4·0	13·0	Pomerat and Willmer (1939)	,,	3·3
Sodium cyanide	35·0	10·0	,,	,,	0·3
Sodium fluoride	20·0	28–84	,,	,,	1·4–4·2
Sodium iodoacetate	0·4	1·0	,,	,,	2·5
Urethane	650	1000	Bastrup-Madsen (1949)	Damage to dividing cells	1·5
Methyl bis (chlorethyl) amine (nitrogen mustard)	7·0	10·0–20·0	Fell & Allsop (1949)	Total growth in 48 hr.	1·6–3·0

E.M.C.—Effective mitotic concentration in embryonic tissue.

DISCUSSION.

In the case of the values for E.M.C., the results depend on repeated experiments where serial dilution in steps of two were used. Examination of the papers quoted shows that dilutions by similar or greater steps have been employed. Thus, in all cases the results recorded may indicate a range from half to twice the figure given, so that in comparing two corresponding results a significant

difference must at least lie outside this range. To compare corresponding results Table I records figures obtained by dividing the E.M.C. values into the tissue culture values. The above considerations show that if this figure lies between 0·5 and 2·0 the difference so indicated is not significant, and statistical considerations might widen this range. It will be seen that most of the resulting quotients fall within the range 0·5–2·0. Sodium azide with a value of 3·3, and sodium cyanide with a value of 0·3 depart most widely from this range, but, considering the inaccuracies of the methods used, it cannot be said that a significant difference is indicated in either case.

Thus, in no substance investigated can a significant difference be established between the results obtained by tissue culture methods and those obtained by the present method using isolated embryonic tissue. It is, therefore, possible to suggest that the latter method can be used as an alternative to tissue culture methods in comparing the concentrations of chemical reagents necessary to inhibit cell division.

SUMMARY.

Using brain tissue from the embryonic chick a method is described for measuring the effective concentration of mitotic inhibitors.

Results obtained by this technique do not differ significantly from results obtained by the use of tissue cultures.

This work was performed whilst in receipt of a personal grant from the British Empire Cancer Campaign. The writer is indebted to Miss Eileen Blake for technical assistance.

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TUBULAR NECROSIS FOLLOWING TEMPORARY OCCLUSION OF THE RENAL ARTERY IN THE RAT.

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FOLLOWING the suggestion that the uraemic syndrome in traumatic anuria and allied conditions may be due to renal anoxia, many observers have investigated experimentally the effects of temporary occlusion of the renal vessels. This frequently results in degeneration and necrosis of the renal tubules, but the distribution of these changes has shown wide variation. McEmery, Mayer and Ivy (1926–27) clamped the renal vessels in dogs and noted that the inner zone of the cortex was more frequently damaged than the subcapsular zone. Scarff and Keele (1943) found that in rabbits killed between the 2nd and 4th days following clamping of the renal pedicle, there was marked necrosis of the first portion of the secreting tubules, whilst the distal segments contained casts. Mallory (1947) observed diffuse degeneration and necrosis in the proximal tubules in dogs with renal arteries clamped 2 to 6 hours, though insignificant changes occurred in the lower nephron. van Slyke, Phillips, Hamilton, Archibald, Dole and Emersen (1944) recorded degenerative changes in the distal tubules in dogs after occlusion of the renal artery for 3 hours, but stated that the visible effects in the tubules were not great enough to account for the total renal failure. Badendoch and Darmady (1947) partially occluded one renal artery in rabbits after removal of the opposite kidney, and described several grades of tubular changes related to the duration of life following operation. In rabbits dying within 2 days there was almost complete destruction of the first and second convolutions, but in those dying 7 to 15 days after operation there were minimal changes in the convoluted tubules, but marked degeneration in the ascending limb of Henle. Thus some showed necrosis in the proximal segments of the tubules, others in the distal segments.

The purpose of this paper is to describe the changes in the rat kidney following temporary occlusion of the artery, and to relate these changes to the blood to the different parts of the kidney. The work quoted has shown conclusively that the mortality rate following temporary clamping of the renal artery is directly related to the period of occlusion. Accordingly a period of 90 minutes' occlusion was decided upon, since this was likely to produce lesions in most animals, but unlikely to cause early death.

EXPERIMENTAL METHODS.

General procedure.

White albino rats about 3 months old and 200 g. weight, maintained on a diet of meat cubes and water, were used in the experiment. They were starved on the day of operation.

Operative procedure.

The animals were anaesthetized by 0·15 ml. veterinary nembutal (intraperitoneal) followed by ether. Each kidney was exposed through a separate lumbar incision. The left kidney was first exposed, the renal artery separated from the vein and a V-shaped clip introduced over the artery. The clip consisted of bent silver foil, the limbs being 3 mm. apart. The artery was slipped into the bend of the clip, the ends of which were tied with silk. The kidney was then replaced into the normal position and the lumbar incision closed with a temporary suture. The right kidney was then excised and the incision closed in layers. The animal was allowed to recover. It was then re-anaesthetized with ether and the clip on the artery removed after it had been in position for 90 minutes.

In a further series of rats the right renal artery was completely occluded and the left renal artery partially occluded for varying intervals from 30 to 120 minutes, and the animals killed 24 to 48 hours later.

Biochemical investigations.

The blood urea using 0·1 ml. of blood from the tail was estimated daily by the method of Archer and Robb, using a Lovibond colorimeter.

RESULTS.

Twenty-two rats were successfully operated upon and observed during a period of 10 days, when surviving animals were killed. It was found that the animals could be divided into 4 groups depending upon their period of survival, degree of urea retention and the histological findings.

TABLE I.

Group.	Number of rats.	Survival.	Blood urea.
I	7	Killed 10 days	Slight rise to 80 mg. per cent. Return to normal in 4 to 5 days.
II	8	Ditto	Moderate rise to 250 mg. per cent. Return to normal in 8 to 10 days.
III	3	2–4 days	Rapid rise to > 360 mg. per cent.
IV	4	6–9 ,,	Rapid rise to > 300 mg. per cent. Persistent high level until death.

Histology.

Group I.—No changes were noted in these rats killed on the 10th day after operation.

Group II.—Occasional calcified tubules were seen in the outer stripe of the medulla, but no other changes were observed.

Group III.—There was widespread coagulative necrosis of the tubules (except collecting tubules) in the outer stripe of the medulla, extending into the inner stripe. The cortex showed extensive irregular areas of degenerated convoluted tubules up to complete necrosis (Fig. 1). In one rat the necrosis was localized to the outer stripe of the medulla (Fig. 2). Tubules not necrosed frequently showed fatty change. The collecting tubules and loops of Henle contained casts.

Group IV.—The lesions were almost entirely localized to the outer stripe of the medulla, where the necrosed tubules were undergoing calcification (Fig. 3). Regeneration of tubules could be seen.

The vulnerability of the tubules in the outer stripe of the medulla to ischaemic necrosis was confirmed in the series of rats with occlusion of both renal arteries, which were killed after 24 and 48 hours. With complete occlusion of the renal artery the necrosis was usually more extensive than with partial occlusion.

The variability of the lesions may be partly explained by the crude technique in constricting the renal artery, and partly by the fact that vascular spasm may persist for a varying time after removal of the clamp.

DISCUSSION.

It is evident that in the rat the tubules in the outer stripe of the medulla are particularly liable to necrosis when the blood flow through the kidney is partially interrupted. The tubules in this zone are the terminal segment of the proximal convoluted tubule and the ascending limb of Henle (Peter, 1909). Suzuki (1912) commented that these two segments were quite similar both in diameter and epithelium (though differentiated by a brush border) and hence could be easily confused. When tubules are necrosed or calcified it is especially difficult to be certain which segment is involved. However, in many cases only collecting tubules are seen intact, and it is evident that both proximal convolutions and ascending limb of Henle are necrosed. Necrosed limbs of Henle can at times be seen passing into the inner stripe of the medulla. The appearances suggest that neither the proximal convolution nor the limb of Henle are selectively damaged, but that all tubules (except collecting tubules) in the outer stripe tend to be involved. The necrosis is thus of a zonal type.

The zonal nature of the lesion with involvement of two segments of the nephron suggests that the necrosis is not due to particular vulnerability of the tubules to oxygen lack, but to deficient blood flow through the outer stripe of the medulla. Badendoch and Darmady (1947) state that Dunn and Polson (1926) demonstrated that the limbs of Henle in the rabbit kidney are the last to be supplied with blood, but we have been unable to trace this reference. Trueta, Barclay, Daniel, Franklin and Prichard (1947) by means of angiographs

EXPLANATION OF PLATES.

FIG. 1.—Rat kidney showing complete coagulative necrosis of secretory tubules in cortex and outer zone of medulla. Glomeruli and collecting tubules intact. Rat dying 48 hours after temporary occlusion of renal artery. H.E. × 65.

FIG. 2.—Rat kidney showing zonal necrosis localized to outer stripe of medulla. Rat dying 48 hours after temporary occlusion of renal artery. H.E. × 65.

FIG. 3.—Rat kidney showing necrosed calcifying tubules localized to outer stripe of medulla. Rat dying 8 days after temporary occlusion of renal artery. H.E. × 65.

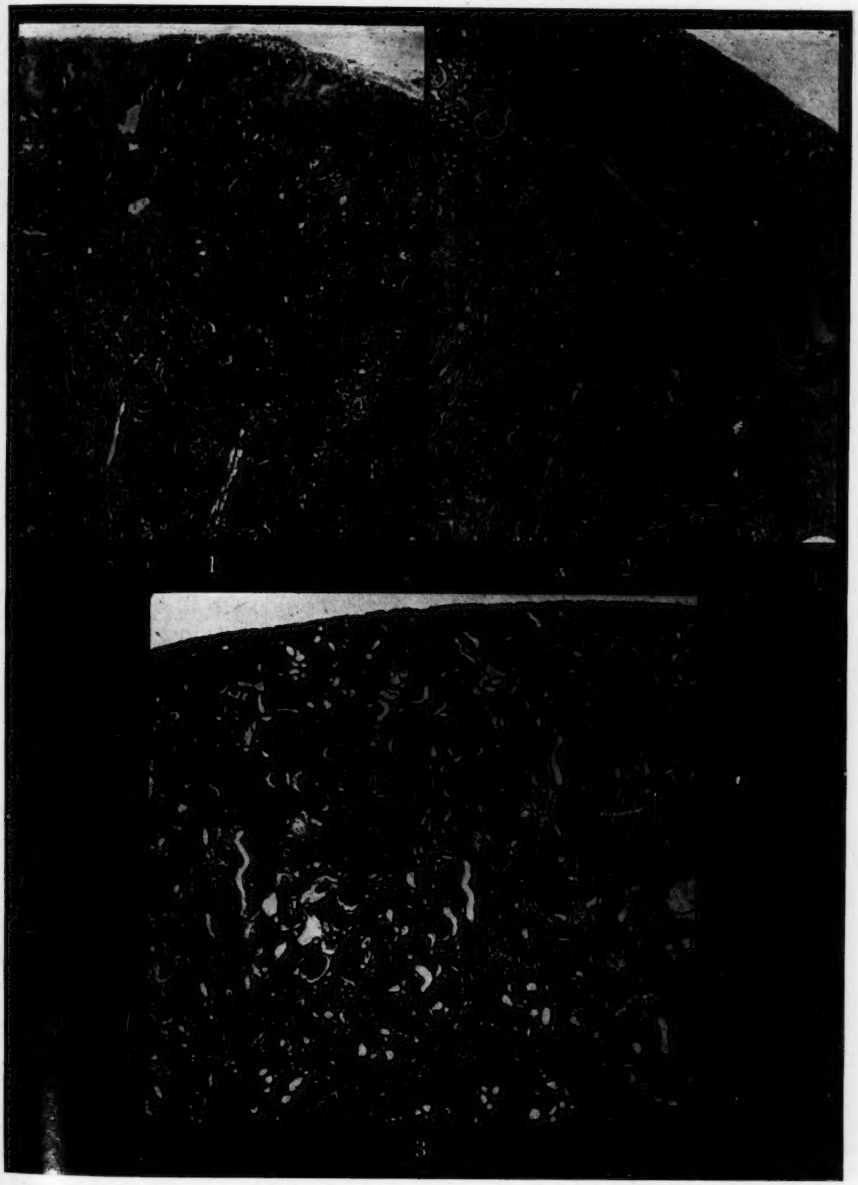
FIG. 4.—Normal rat kidney injected with India ink via thoracic aorta during life. Note lack of injection of vessels in outer stripe medulla. Frozen 300 μ. × 3.

FIG. 5.—Perfused rat kidney injected with India ink at 200 mm. Hg via renal artery. The fine network of vessels in outer stripe of medulla is seen; these are less injected than other zones of kidney. Frozen 300 μ. × 3.

FIG. 6.—Perfused rat kidney injected with India ink 20 mm. Hg via renal vein. Note the failure of injection of vessels in outer stripe of medulla. Frozen 300 μ. × 3.

FIG. 7.—Rat kidney following mercuric chloride (0·01 g./kilo body weight) showing calcification (black) of necrosed tubules in outer stripe of medulla. von Kossa. × 3.

FIG. 8.—Rat kidneys injected with India ink via thoracic aorta during life at hourly intervals following subcutaneous mercuric chloride. Note poor injection of kidneys returning to normal at 4 hours. × 2.



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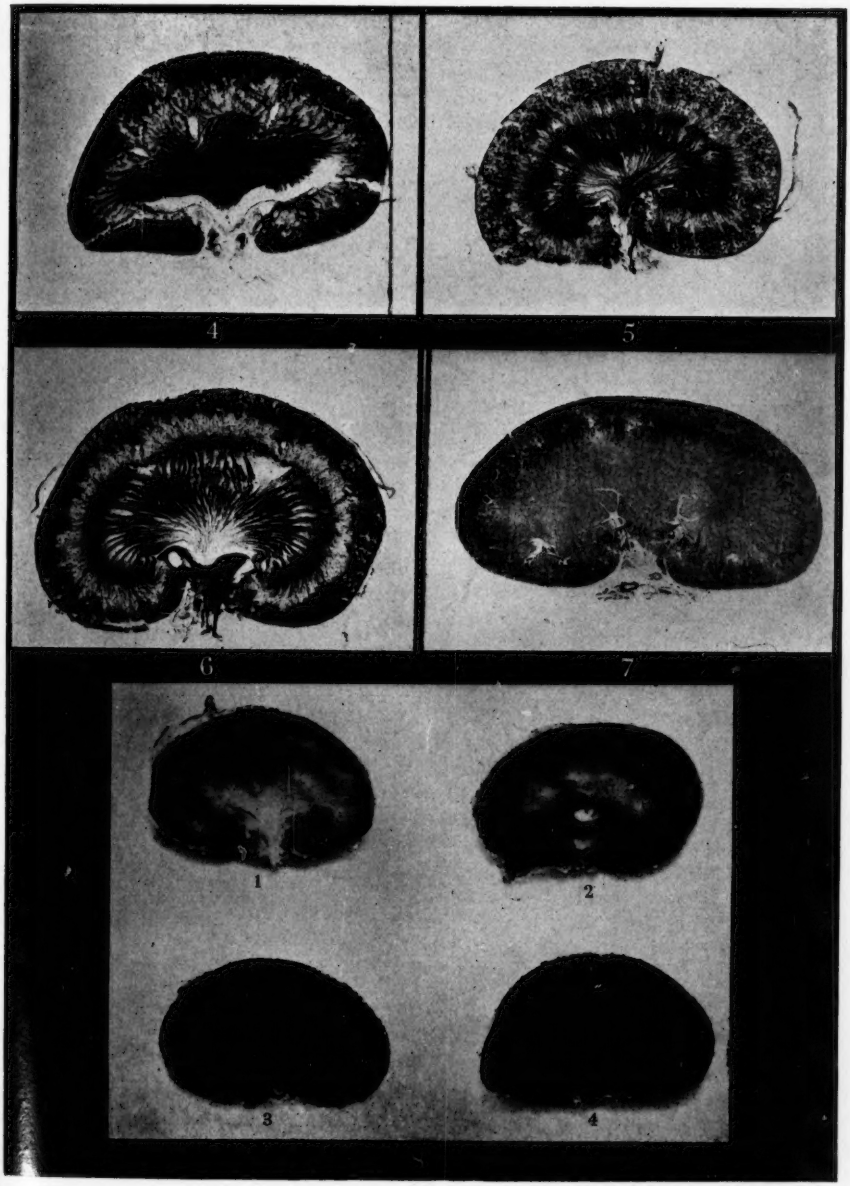

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in normal rabbits have described arterial and venous stages of filling of the kidney. In the late arterial stage the maximal filling was in the cortex, whilst in the early venous stage the maximal shadow was in the medulla, and especially the subcortical zone. They considered these distinct stages indicated a slow blood flow through the medullary channels.

Though there is a complex meshwork of vessels in the outer stripe of the medulla of the rat kidney (Trueta *et al.*, 1947) it is very difficult to inject these vessels during life and in the isolated kidney. We have injected Indian ink (filtered and diluted 1 in 2 with saline) into the thoracic aorta during life, and into the renal artery and vein of the perfused kidney. In animals injected via the thoracic aorta the cortex rapidly becomes black and the inner zone of the medulla and the inner stripe of the outer zone are injected, but little filling is seen in the vessels in the outer stripe of the medulla (Fig. 4). This appears to indicate a slow flow of blood through these vessels. Fig. 5 shows a perfused kidney injected via the renal artery and the poor filling of the vessels in the outer stripe is clearly seen. The lack of filling in this zone is even more marked following venous injection (Fig. 6). This may be due to the way in which these vessels terminate in the larger veins, so that a rise in venous pressure may obliterate the openings of the venous plexuses. Such a mechanism could further slow the circulation through the outer stripe of the medulla.

It thus appears that the blood flow through the outer stripe of the medulla is very slow, and if the circulation through the kidney is diminished, then one would expect the maximal effects from anoxia to occur in this zone. Their functional activity and oxygen requirements at that particular time, may determine which tubules undergo necrosis.

It is of interest to relate this zonal necrosis to the lesions found in traumatic anuria and allied conditions in man. Various authors have stated that the most marked lesions were found in the "boundary zone." Mallory (1947) observed that the lesions were maximal in a narrow zone about 2 mm. wide on the medullary side of the cortico-medullary junction. This corresponds with the outer stripe of the medulla, though the division of the medulla into zones is not as well defined in man as in animals. This location suggests that the tubular necrosis in traumatic anuria is produced by renal ischaemia. Characteristically in traumatic uraemia the naked eye picture shows extreme congestion of the medulla (especially sub-cortical) with a pale and swollen cortex. Trueta, *et al.* (1947) suggested that this indicated a shunt of blood from the cortical to the medullary vessels. If this were true, then the outer part of the medulla would have a better blood supply than the cortex, and one would not expect to find necrosed tubules in this zone. It seems more likely that the injection of the medulla indicates a passive congestion with a very slow blood flow.

It has long been recognized that mercuric chloride produces necrosis of tubules, particularly in the outer stripe of the medulla (Oliver, 1915), but opinions varied as to the exact segment of the nephron involved. Lyon (1904) considered the ascending limb of Henle to be particularly damaged; others that the terminal segment of the proximal convolution was chiefly involved (Oliver, 1915). As noted earlier, these tubules are somewhat similar and can easily be confused. It is also known that the tubular damage is very variable and may involve the convolutions in the cortex. It is generally considered that mercuric chloride exerts a specific toxic effect on certain segments of the renal tubule. However,

MacGraith, Havard and Parsons (1915) suggested that the toxic effect of certain heavy metals may be related to renal anoxia.

We have examined the effect of mercuric chloride on the rat kidney. With minimal dosage (0·01 g./kg. body weight), necrosis of tubules is almost entirely limited to the outer stripe of the medulla, and apparently involves all the tubules (excepting collecting tubules) in this zone. Larger dosage produces variable necrosis in the cortex, but the effect is not directly proportional to the dosage. This fact suggests an indirect mechanism. Calcification of the necrosed tubules (Fig. 7) is common just as occurs following clamping of the renal artery. If mercuric chloride is given intravenously a sudden pallor of the surface of the kidney is noted. This is due to spasm of the renal artery and not to diversion of the blood through the medulla. A number of rats were given mercuric chloride subcutaneously in a dosage which would cause marked necrosis in the outer stripe. The rats were then anaesthetized at half-hourly intervals, the thorax opened and Indian ink injected into the thoracic aorta. There was an irregular injection of the kidneys, maximal after 1 hour, which persisted for 2 to 3 hours following the mercuric chloride (Fig. 8). This clearly indicates a diminished blood flow through the kidney for a variable period. These facts suggest that toxic action of mercuric chloride on the kidney is at least in part an indirect mechanism due to the production of temporary renal ischaemia.

SUMMARY.

Temporary occlusion of the renal artery in the rat produces necrosis of tubules, often entirely localized to the outer stripe of the medulla.

It is considered that this zonal necrosis is related to the slow blood flow through this region of the kidney.

The similarity of this ischaemic necrosis to the necrosis produced by mercuric chloride is emphasized. It is suggested that the action of mercuric chloride is at least in part due to the production of temporary renal ischaemia.

I wish to thank Professor S. L. Baker for helpful advice and criticism, and Mr. F. Ward for the photomicrographs.

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THE ABSORPTION OF OLEIC ACID FROM THE INTESTINE OF RATS DEFICIENT OF FOLIC ACID AND BIOTIN.

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RECENT work suggests that sprue may be a deficiency disease (Stannus, 1942 ; Leishman, 1945 ; Ayrey, 1947 ; Angulo and Spies, 1947). Stannus (1942) suggested that lack of B_2 vitamins in the intestine leads to failure of absorption of fatty acid, and that this is the fundamental defect in sprue. Accordingly it seemed important to study the effect of vitamin-deficient states on the absorption of fatty acids. Mottram *et alia* (1922) studied the intestinal mucosa of rats after giving them fat to which " Marmite " had been added. Otherwise no detailed studies on the effect of vitamins on fat absorption had been made until Frazer (1949) observed histologically the bowels of rats given mixtures of olive oil, and thiamine, riboflavine, nicotinamide, pyridoxine, pantothenic acid and choline. Only choline appeared to increase the rate of fat absorption. Black, Bound and Fourman (1947) showed that nicotinic acid and riboflavine are apparently without effect on fat absorption in sprue. The present investigation concerns folic acid and biotin, two important members of the vitamin B_2 complex not covered by these previous studies. In this connection folic acid appears to be of particular interest in view of its therapeutic effect in sprue.

METHODS.

Animals.

Black and white weanling rats of the National Research Institute strain were fed *ad libitum* on purified rations consisting of 66 per cent starch, 18 per cent casein, 9 per cent arachis oil, 5 per cent salt mixture (Jones and Foster, 1942), and 2 per cent sulphasuccidine. Only animals weighing from 30 to 42 g. at the commencement of the experiment were used. They were kept in individual cages, and in order to prevent coprophagy the floors of the cages were raised $\frac{3}{4}$ in. from the bench, and consisted of $\frac{1}{2}$ in. open wire mesh. No straw was placed in the cages.

Vitamin supplements.

It has been shown (Wright and Welch, 1944) that effects attributable to deficiency of folic acid and biotin arise when rats are given purified rations containing 1 to 2 per cent sulphasuccidine and adequate amounts of thiamine, riboflavine, nicotinic acid, pyridoxine, pantothenic acid, inositol, *p*-aminobenzoic

acid, and choline. All the animals were given a daily supplement of 10 international units vitamin A ; 5 international units vitamin D, and menaphthone 5μg. They were divided into four groups and received other vitamins as follows : (i) All vitamins as under-control group ; (ii) all vitamins less folic acid ; (iii) all vitamins less biotin ; (iv) all vitamins less folic acid and biotin. The animals in the control group received the following daily vitamin supplements : Choline chloride 3 mg., inositol 1 mg., *p*-aminobenzoic acid 1 mg., aneurine 15 μg., riboflavine 40 μg., pyridoxine 10 μg., sodium-D-pantothenate 50 μg., nicotinamide 200 μg., biotin 1 μg., ascorbic acid 500 μg., folic acid 20 μg.

An aqueous solution of the vitamins was prepared, of which 1 c.c. contained the daily supplement. This amount was added to the drinking water each morning, the animals being supplied with water from inverted bottles equipped with feeding tubes of standard type. Sufficient vitamin solution was prepared to last for a month ; it was stored in the dark at 2° C.

EXPERIMENTAL PROCEDURE.

At the end of a $2\frac{1}{2}$ to 4 months' period the animals were starved for 24 hours, anaesthetized by the intramuscular injection of 2 per cent urethane in normal saline, and 1 c.c. of oleic acid was introduced into the stomach through a cannula passed down the oesophagus. After $6\frac{1}{2}$ hours the animal was killed and the gastro-intestinal tract removed and divided into stomach, small intestine, and caecum with colon. These were washed out separately with tap water, and the fat content of the washings estimated by the standard technique using a Soxhlet apparatus and extracting with ether, this procedure being based on that of Frazer.

From the figures obtained the amounts of oleic acid absorbed per hour were calculated. An unusually large quantity of fat present in the caecum would afford supporting evidence of failure of absorption in the small intestine. The amounts of fat recovered from the caecum at the end of absorption experiments were therefore estimated, and compared with the amounts recovered from control animals killed after 24 hours' starvation.

This procedure was adopted after previous experiments had given inconclusive results. In these chylomicron counts had been used to assess fat absorption.

RESULTS.

Absorption of oleic acid in animals deficient of folic acid and biotin.

It was found that if a rat is starved for 24 hours and then killed, the fat present in the caecum and colon is similar in quantity to that recovered at the end of fat absorption experiments carried out as above described (Table II). This implies that during the period of the experiment little or no fat reaches the caecum from the stomach. In calculating the amount of fat absorbed during the experiments, therefore, it seems that the caecal fat should be ignored. The absorbed fat would then equal the amount given to the animal less that recovered from the stomach and small intestine only. By subtracting the amount of fat which was recovered from the stomach from that originally administered, the amount which had left the stomach and which was therefore available for absorption was estimated in mg. The amount absorbed is expressed as the percentage of this quantity absorbed per hour.

It is seen (Table I) that the amounts of oleic acid absorbed do not differ significantly in the respective groups. This is confirmed by the application of the "t" test of significance. In particular there is no evidence that deficiency of folic acid or biotin impairs the absorption of oleic acid.

For purposes of comparison with the values given in Table I, the following are the figures calculated on the basis of the absorbed fat being equal to the amount given to the animal less that recovered from stomach, small intestine, caecum and colon. Absorption of oleic acid reaching the small intestine (percentage absorbed per hour) : Control group, mean 8·17, standard deviation (S.D.) ±1·36 ; folic acid deficient animals, mean 7·60 ; S.D. ±2·46 ; biotin deficient animals, mean 7·83, S.D. ±0·74; folic acid and biotin deficient animals, mean 7·50, S.D. ± 1·68. The differences are not such as would alter the conclusions reached.

TABLE I.—*Oleic Acid leaving Stomach and therefore Available for Absorption in Various Groups of Animals ; Amounts Absorbed, and Amounts Recovered from Caecum and Colon at End of Experiments.*

	Amount of oleic acid leaving stomach during experiment (mg. per hour).		Absorption of oleic acid reaching small intestine (percentage absorbed per hour).		Fat recovered from caecum and colon (mg.).		Number of animals experimented upon.	Mean weight of animals (g.).
	Mean.	Standard deviation.	Mean.	Standard deviation.	Mean.	Standard deviation.		
Control group (animals receiving full vitamin supplements)	48·0	±18·65	10·84	±1·36	52·0	±4·30	4	108·0
Folic acid deficient animals	60·4	± 5·49	9·65	±2·18	53·2	±28·20	4	97·0
Biotin deficient animals	47·9	±26·39	10·35	±0·74	40·2	±12·10	4	97·5
Folic acid and biotin deficient animals	42·5	±39·60	9·66	±2·82	27·7	±17·16	4	72·5

Fat in caecal and colonic contents of control rats killed immediately after a 24-hour period of starvation.

Six rats were killed after such a period. For eight or more weeks they had been on synthetic diets identical with those of the other experimental animals. Full vitamin supplements as in the control group had been given to two of the rats, while two were deficient of biotin and two of folic acid and biotin. The stomachs and small intestines of these animals were empty at death. The size of the caecum was roughly proportional to the weight of the animal. The amounts of fat recovered from the caecal and colonic contents are shown in Table II.

It is seen that—

(i) These amounts are roughly proportional to the animal's weight. Differences in the weight of an animal at the time of the experiment therefore would lead to error if the fat in the caecal contents was regarded as having been derived from that introduced experimentally into the stomach.

(ii) The quantities of fat do not differ significantly from those recovered

from the caecal and colonic contents at the end of the absorption experiments (Table I).

It seems likely that the 24-hour period of starvation preceding these experiments causes only partial emptying of the large bowel, the majority of the fat remaining in the bowel probably being derived from food previously taken, supplemented by small quantities which have been excreted by the bowel mucosa. It appears that little if any of the fat introduced into the stomachs of the animals normally reaches the caecum during the 6½-hour period of the experiment.

TABLE II.—*Fat in Caecal and Colonic Contents of Control Rats Killed immediately after a 24-hour Period of Starvation.*

	Animals individual weight (g.).	Fat recovered from caecum and colon (mg.).	Mean of the six observations (mg.).	Standard deviation.
Animals receiving full vitamin supplements	70·0	27·0	47·58	±14·50
	121·0	55·0		
Biotin deficient animals	76·0	30·5		
	116·5	50·0		
Folic acid and biotin deficient animals	81·0	54·5		
	115·0	68·5		

Fat in caecal and colonic contents of animals at conclusion of absorption experiments.

The amounts recovered from the controls having full vitamin supplements did not differ significantly from those in the vitamin deficient groups (Table I), this being confirmed by the " t " test. In the group deficient of folic acid and biotin the amounts were smaller than in the other groups, but as the mean weight of the animals was proportionately smaller in this group than in the others it is unlikely that the reduced figure is related to alteration in the rate of fat absorption.

It is conceivable that no increase in caecal fat would occur if fat was being absorbed inadequately and at the same time intestinal motility was impaired. If, however, the amount of fat leaving the stomachs of the animals can be taken to indicate the degree of intestinal motility, this would seem to be similar in each group (Table I). Application of the " t " test reveals that the amounts of fat leaving the stomachs of the animals in the various groups do not differ significantly. In no group did an unduly large amount of fat reach the caecum during the experiments. This observation therefore affords additional evidence that there was no impairment in absorption of oleic acid in any group of animals.

DISCUSSION.

Fat excreted into the intestine is a possible source of error in absorption experiments of the kind described (Hill and Bloor, 1922). Sperry (1926), however, showed that dogs of 5 to 13 kg. weight excrete only 1·5 to 2 g. non-volatile lipids weekly. It seems unlikely therefore that rats of 100 g. weight would excrete more than 0·200 g. weekly, or 0·003 g. during an experiment of 6½ hours' duration. This quantity would produce a small constant error which would not alter the main conclusions drawn from the experiments. Sperry (1926) also stated that it is likely that the fat is excreted from the mucosa lower down the intestine than the fat-absorbing surface. It is probable therefore that by excluding the fat recovered from caecal contents in calculating the fat absorbed this source of error is avoided.

On the basis of the evidence presented it seems unlikely that either folic acid or biotin is directly concerned with the absorption of oleic acid from the intestine of the rat. Attention has been drawn elsewhere (Woodruff, 1950) to the failure of folic acid to influence fat absorption in the sprue syndrome in man. In so far as the results of animal experiments with a single fatty acid are applicable to man, this study further supports the view that a deficiency of folic acid is not responsible for the increased amounts of fatty acid found in the stools of patients with sprue.

SUMMARY.

Rats were maintained on a standard diet, and to a series of controls and to others deficient of folic acid and biotin, 1 c.c. oleic acid was administered. After 6½ hours the distribution of fat in the intestine was estimated.

The amounts of oleic acid absorbed after reaching the small intestine varied in the different groups from 9·65 to 10·84 per cent per hour.

The amounts of fat recovered from the caeca at the end of the experiments were not significantly increased in any group.

The rate of absorption of oleic acid, therefore, in animals deficient of folic acid and biotin was not found to be significantly different from that in control animals.

It is suggested that the increased quantities of fatty acid found in the stools of patients with sprue are unlikely to result from deficiency of biotin or folic acid.

I wish to thank the University of Durham for a research grant with which the earlier part of this work using chylomicron counts was done; with the help of my wife this portion was carried out in the Department of Physiology, King's College, Newcastle-upon-Tyne. I also wish to thank Dr. F. Murgatroyd, Dr. C. C. Ungley, Dr. J. Martin and Dr. D. S. Ridley for their comments; and Dr. T. F. McCrae for advice regarding the rats' daily vitamin supplements.

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THE EFFECT OF BACTERIA, BACTERIAL AND OTHER POLYSACCHARIDES, AND VARIOUS MACROMOLECULAR SUBSTANCES ON INFLUENZAL INFECTION IN MICE AND ON HAEMAGGLUTINATION BY INFLUENZA VIRUSES AND CHEMICAL AGENTS.

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HORSFALL and McCarty (1947) found that a number of bacterial and other polysaccharides exerted a striking modifying effect on infections with the pneumonia virus of mice. This discovery followed the observation that certain streptococci given intranasally to mice infected with the virus greatly diminished the severity of the ensuing pulmonary lesions, even though the streptococci did not persist in the lung for more than a few hours. The active polysaccharides did not inhibit agglutination of red cells by the virus.

Ginsberg, Goebel and Horsfall (1947, 1948*a*, 1948*b*) noted that the capsular polysaccharides of type-specific Friedländer's bacilli, among the most active in Horsfall and McCarty's experiments, were firmly adsorbed to chicken red cells and prevented their agglutination by mumps and some other viruses. Injected into the allantoic cavity or yolk-sac of the developing egg, they inhibited the growth of mumps virus injected into the allantoic cavity either previously or as long as 4 days afterwards ; as little as 5 μg. polysaccharide were effective. They did not inhibit growth of the virus of Newcastle disease in chick embryos, or of influenza A or B in embryos or mice.

These authors further showed that inhibition of haemagglutination and of multiplication of virus are not closely related phenomena. Thus, in general terms, polysaccharides which inhibit multiplication of a virus may or may not inhibit haemagglutination by that virus, and, again, may or may not prevent adsorption of virus to red cells. Treatment of Friedländer polysaccharide with OH^- abolishes its power to inhibit multiplication of mumps virus, but leaves haemagglutinating properties substantially intact, while treatment with periodic acid has the opposite effect. It is improbable, therefore, that the erythrocytic component reacting with virus is closely related to the cellular constituents responsible for multiplication of virus. Concerning the latter, evidence was obtained that the so-called virus-receptors are not blocked by polysaccharide ; in its presence, virus is adsorbed normally by the cells of the living allantoic

membrane, and it is some later step in the multiplication of virus which is prevented.

Green and Woolley (1947) have shown that a number of simple and complex carbohydrates inhibit the agglutination of chicken red cells by influenza virus, and that one (apple-pectin) prevents multiplication of virus in the chick embryo. Woolley (1949) obtained from erythrocytes a substance probably of polysaccharide nature; this substance antagonized the effect of apple-pectin on haemagglutination by influenza virus, and was itself destroyed by the virus *in vitro*. These observations suggested, without finally proving, that apple-pectin and certain other polysaccharides inhibit haemagglutination and multiplication of virus by retarding an enzymic attack by the virus on a structurally similar polysaccharide in the cell.

It will be seen that at a number of points in this work hints of possible therapeutic action occur, and it was from this aspect that we began experiments along the same general lines, at a time before several of the above reports had been published. Our experiments may be considered under three separate but interrelated headings: (*a*) examination of bacterial cultures for power to modify influenzal infection in mice, (*b*) a survey of polysaccharides and other large molecules for ability to inhibit haemagglutination by virus and other agents, and (*c*) examination of macromolecular substances for a protective effect against influenza in mice.

METHODS.

Influenza in mice.

Groups of 10 randomized mice (20 g. $\pm$ 1 g.) received into each nostril one drop of a dense bacterial suspension, or of a solution of polysaccharide or other compound, 4 hours before instillation by the same route of a 10^{-3}, 10^{-5} or 10^{-6} dilution in horse-serum saline of influenza virus A (PR8 strain). Controls were treated with uninoculated culture medium or with water, respectively. The pipettes used delivered approximately 34 drops per c.c. In many cases the solution of polysaccharide was of a strength of 1 per cent, in which event the animals received about 0·6 mg. polysaccharide. In a few tests we repeated the intranasal instillation of polysaccharide 24 and 48 hours after virus; although usually we did not thereby enhance the severity of the influenzal lesions, as noted by Taylor (1941), we certainly did not obtain more favourable results from repeated administration.

Initially we killed survivors on the 7th day (" 7-day test " in Table II) and scored the animals as follows: 6 = death with influenzal lesions on or before the 5th day; 5 = death with influenzal lesions on the 6th or 7th days; 4 to 0 = survival with various degrees of pulmonary consolidation. We then calculated the mean pulmonary scores for each group. In attempted chemotherapy by other means, however, we found that effects apparently beneficial when judged in this manner might, in duplicate groups of mice, be accompanied by no increase in the mean period of survival, or even by reduced survival. In later work, therefore, we infected duplicate groups of mice. Survivors of one group were killed on the 7th day and the group scored as above. Of the other group we allowed such animals as would to survive until the 21st day, taking both the mean pulmonary score and the mean period of survival as criteria of the effect of treatment (" survival test "). Subsequent experience in groups of control mice showed that

the mean pulmonary scores on the 7th and 21st days rarely differed significantly. In consequence we have recently used only the survival test as affording ordinarily all the information obtainable from both tests.

Inhibition of haemagglutination caused by influenza viruses or by chemical agents.

On the ground of greater reliability we ordinarily performed haemagglutination tests according to the technique of Hirst and Pickels (1942). However, anomalous reactions in the presence of some of the polysaccharides were sometimes recognized more easily by the direct visual method ; we checked the results, therefore, by the pattern test (Salk, 1944) and, on occasion, by examination with the microscope.

Red cells.—Thrice-washed red cells of individual fowls were used in an initial concentration of approximately 1 per cent, as more suited to our photoelectric colorimeter than the usual concentration of 1·5 per cent. In every test we adjusted the concentration so that a 1 in 4 dilution gave a standard reading on the instrument (± 5 per cent). Cells collected on a Monday served until the following Friday ; the probable deterioration of cells during this period can be gauged from the observations of Miller and Stanley (1944).

Virus.—Allantoic fluid infected with the PR8 strain of influenza A or the Lee strain of influenza B was titrated (6 replicates) against each new batch of red cells, and 4 HU (haemagglutinating units)were used in the test. Virus heated at 56° C. for half an hour was similarly titrated and used in simultaneous tests with living virus. Virus was passaged every 3 weeks or so, and that for use in the test was kept with 1:10,000 merthiolate at + 2° C.

Nitroakridin 3582.—Nitroakridin also was titrated against each new batch of red cells. Compared with influenza virus it gives a curve with a very steep slope. With some of the polysaccharides, the stronger solutions gave precipitates. In the tests, therefore, we used ordinarily only 1 HU nitroakridin, as often a solution of this strength did not precipitate whereas one containing 4 HU did; if necessary, and if possible, we confirmed the result with 2 or 4 HU. We made a suitable correction for the colour of the nitroakridin.

Phaseolus vulgaris.—Extracts made by the method of Li and Osgood (1949) were titrated against each batch of red cells. The agglutinated masses were very small, and the slope of the curve more gradual than with influenza virus.

Polysaccharides, etc.—The sources or methods of preparation of these are dealt with in an appendix. We studied the effect of successive two-fold dilutions starting with a concentration of 2000 μg./c.c. When the pH of the solution lay outside the range 6·0 to 8·0 we carried out parallel tests with neutralized and unneutralized preparations. Not all the solutions were perfectly clear, and in high concentration some themselves agglutinated or otherwise affected the red cells.

Conduct of the test.—Ordinarily 0·5 c.c. virus was added to successive dilutions (0·5 c.c.) of polysaccharide, the mixture allowed to stand half an hour at room temperature (22–26° C.), after which the red cells (1 c.c.) were added vigorously from a Cornwall pipette. Readings were taken after 60 minutes in the Hirst test, and after 2 hours or more in the Salk test. Three replicates of each row of tubes comprised a single test. Controls included saline and red cells ; compound, saline and red cells ; and virus, saline and cells.

In the tests with nitroakridin, this substance was added last with a very

vigorous action to avoid excessive local concentration. It may at once be stated that differences between the tests with virus and those with nitroakridin were not due to the different order of adding the various components. In most cases, with virus no difference was seen whether virus or blood were added first and allowed to stand half an hour before addition of the other component. The few exceptions are not relevant to the point under discussion.

Tests with bean extract were conducted similarly to those with virus.

EXAMINATION OF BACTERIA FOR AN EFFECT ON INFECTIONS IN MICE DUE TO INFLUENZA VIRUS A.

These experiments were designed to show whether or not an effect similar to that described by Horsfall and McCarty in mouse-pneumonia could be demonstrated in influenzal infections of mice.

None of over 160 bacterial species obtained from the National Collection of Type Cultures, or freshly isolated from soils by our colleague, Dr. A. R. Martin, had a significantly beneficial effect when instilled intranasally 4 hours before virus. Although the suspensions used were very dense and quite opaque, surprisingly few (mostly of recognized pathogens) produced an adverse effect. Since this attempt was made, Ginsberg and his colleagues (1948*a*) have reported similar negative results against influenza.

EXAMINATION OF POLYSACCHARIDES AND OTHER MACROMOLECULAR COMPOUNDS FOR POWER TO INHIBIT HAEMAGGLUTINATION BY VIRUS OR BY CHEMICAL AGENTS.

Although, as stated in the introduction, the latest work of Ginsberg and his colleagues revealed no close relation between inhibition by polysaccharides of viral haemagglutination on the one hand, and inhibition of the growth of virus (i.e. possible chemotherapeutic effect) on the other, we continued the observations we were making on the effect of macromolecules in the haemagglutination test. Not all workers believe that inhibition of viral haemagglutination by polysaccharides necessarily implies a structural similarity between the inhibitors and the cellular constituent on which the influenza virus makes its enzymic attack. If it were shown that the polysaccharides in question also inhibit agglutination due to an agent quite different from virus, one would have to conclude either that one had disclosed a remarkable and unsuspected similarity of action on the red cell of two very different agglutinating agents, or more probably that the protective action of the polysaccharides is due to some non-specific factor such as " plating " of the red cells. Were macromolecules of very diverse chemical types equally effective as inhibitors, the latter supposition would receive additional support. On the other hand, evidence of a specific relationship existing between virus and polysaccharide might suggest that the latter is chemically or structurally related to the " virus-receptors " of the red cell and competes with these for available virus.

In this work we examined 77 chemical substances for an inhibitory effect on haemagglutination by influenza A or influenza B virus, or both. As evidence of a specific relationship between virus and polysaccharide, we accepted the greater inhibitory effect noted when heat-inactivated virus is substituted for fully-living virus (Francis, 1947). Most of the 77 compounds were also examined for an

TABLE I.—*Effect on Haemagglutination*

Number and identity of compound.	Properties of the solution.	Living influenza A virus, 4 HU.
3. Blood-group O polysaccharide (crude human gastric mucin)	..	S > 2000 (125–
4. Serum-mucoid (human)	..	C 125 (32–2000
5. Ovarian-cyst polysaccharide	Compound itself causes slight agglutination of red cells	C 63 (8–2000
6. Ovarian-cyst (malignant) polysaccharide	..	P > 1200 (125–
7. Hyaluronic acid from umbilical cord (degraded)	..	O
8. Hyaluronic acid fibres	Solution very viscous and greatly delays settling of red cells	C 350 (63–2000
10. Ovomucoid	Solution, pH 4·0, discolours and agglutinates red cells	P > 1300 (250–
Ditto—neutralized	Solution turbid but does not agglutinate red cells	S > 2000 (250–
11. Liver polysaccharide (human)	..	S > 2000 (250–
16. Sheep pituitary mucopolysaccharide	..	S > 2000 (500–
21. Pneumococcus type I specific polysaccharide	Solution turbid and rather viscous. Agglutinates red cells	O
22. Pneumococcus type II specific polysaccharide	Solution viscid and causes some agglutination of red cells	P > 1500 (250–
23. Pneumococcus type III specific polysaccharide	Solution turbid, pH 5·0, discolours and agglutinates red cells	C 100 (32–250
24. Pneumococcus type V specific polysaccharide	Solution causes slight agglutination of red cells	P > 1000 (125–
28. *Azotobacter* polysaccharide	Solution turbid and very viscid, greatly delays settling of red cells	C 250 (8–500
30. *Betacoccus* Birmingham dextran	Solution rather viscous	P > 2000 (250–
32. *Penicillium luteum* polysaccharide	Solution turbid, slightly viscous, pH 4	P > 1000 (250–
Ditto—neutralized	..	O
35. Alginic acid	Solution pH 6·0	S > 2000 (250–
Ditto neutralized	..	..
53. Sodium carboxymethyl ether of cellulose—ratio unknown	Solution rather viscous and agglutinates red cells	S > 2000 (125–
54. Sodium carboxymethyl ether of amylopectin—B.V. 0·008, ratio 1–1·0	Solution viscous	O
55. Sodium carboxymethyl ether of starch—B.V. 0·026, ratio 1–2·2	Extremely viscous. A glycerin-like layer settles out	O
59. Oxycellulose (N_2O_4 method)	Solution slightly viscous	O
60. Polyvinyl alcohol—high viscosity, fully hydrolysed	..	P > 1300 (125–
63. Triglycoladipamide	..	O
65. Acrylamide-methacrylic acid interpolymer	..	O
68. Poly-methacrylic acid	Solution pH 8·5	O
Ditto—neutralized	..	..
69. Desoxyribonucleic acid (thymus)	..	O
71. γ Globulin (human)	Compound causes some agglutination of red cells	C 250 (32–200

C, P, S, O = Various degrees of apparent inhibition of haemagglutination.

For the Hirst test, C = readings characteristic of 100 per cent of red cells in suspension in one or more tu containing the highest concentrations of compound.

P = 50–100 per cent. S = less than 50 per cent of red cells in suspension.

O = No difference from virus + red cell control (or nitroakridin + red cells).

The first figure in each column gives the concentration of compound (μg./c.c.) in the tube in which 50 per cent

arious Substances of High Molecular Weight.

Hirst test : inhibitory action in the presence of				Salk test : inhibitory action in the presence of	
Heated influenza A virus, 4 HU.	Living influenza B virus, 4 HU.	Heated influenza B virus, 4 HU.	Nitroakridin 3582, 1 HU.	Living influenza A virus, 4 HU.	Nitro-akridin 3582, 1 HU.
> 63 (4–500)	S > 2000 (250–)	C 63 (4–250)	C 32 (8–250)	P	C
125 (4–2000)	C 125 (16–2000)	C 8 (1–250)	C 32 (4–250)	C	C
32 (4–2000)	S > 2000 (32–)	P > 2000 (4–)	C 125 (8–250)	P	P
> 900 (125–)	S > 2000 (63–)	P > 1000 (63–)	C 32 (2–250)	C	C
	O	S > 2000 (125–)	C 4 (0·5–16)	O	C
350 (63–2000)	..	..	C 2 (1–16)	P	P
> 1300 (250–)	S > 2000 (125–)	S > 2000 (125–)	P > 1800 (250–)	P	S
> 2000 (250–)	..	..	..	P	S
> 2000 (250–)	S > 2000 (250–)	P 500 (63–2000)	C 10 (2–32)	P	C
> 2000 (500–)	..	..	C 40 (8–125)	S	C
	..	..	C 12 (2–125)	S	P
> 1500 (250–)	P > 1500 (250–)	P > 1500 (250–)	C 6 (1–16)	P	P
100 (32–250)	C 800 (125–2000)	C 800 (125–2000)	..	O	O
..	P > 1000 (125–)	P > 1000 (125–)	C 4 (1–16)	P	C
250 (8–500)	C 300 (63–1000)	C 300 (63–1000)	C 1 (<1–2)	O	O
> 2000 (250–)	..	..	O	P	O
> 1000 (250–)	..	..	P > 300 (32–)	S	C
	..	..	..	P	C
> 2000 (250–)	S > 2000 (250–)	S > 2000 (250–)	C 3 (1–32)	O	C
..	S > 2000 (250–)	S > 2000 (250–)	C 3 (1–32)	S	C
> 2000 (125–)	S > 2000 (125–)	S > 2000 (125–)	C 1 (<1–2)	O	P
	O	O	C 100 (32–125)	O	C
	O	O	C 11 (2–16)	O	C
	O	O	C 40 (32–500)	O	C
> 1300 (125–)	..	..	C 250 (16–1000)	P	C
	O	O	C 300 (16–2000)	O	O
	..	..	C 40 (2–125)	O	C
	..	..	C 175 (125–250)	O	C
..	..	..	..	O	C
	..	..	P > 1000 (32–)	O	P
250 (32–2000)	C 250 (32–500)	C 650 (63–2000)	P > 1300 (63–)	P	P

s remained suspended ; the figures in brackets show the range of concentrations over which the transition from no full inhibition occurred. As indicated in the text, these readings were sometimes vitiated by turbidity arising from nges in the red cells or from other causes ; the conclusion to be drawn in each case is made clear in the text.

For the Salk test, C = complete inhibition of agglutination n one or more tubes.
P = marked. S = some difference in pattern of sedimented red cells.
O = no difference from virus + red cell control (or nitroakridin + red cells).

inhibitory effect on the agglutination caused by Nitroakridin 3582 (Hurst, 1948). A few were tested against the agglutinin of *Phaseolus vulgaris* (Li and Osgood, 1949).

Results.

The compounds examined varied greatly in their chemical and physical properties, and when combined with such diverse haemagglutinins as virus and nitroakridin it seemed inevitable that highly complex reactions would sometimes ensue. Every reaction had to be carefully observed and considered on its merits; even so, paradoxical results occurred at times, as for example when readings corresponding to marked inhibition of agglutination were obtained in the Hirst test, yet visually and microscopically the red cells were agglutinated. Delayed settling of the agglutinated masses would, of course, account for this observation, and great viscosity of the compound under test provide a facile explanation of its occurrence. As pointed out later, however, there is no certain parallel between viscosity of the solution and the rate of deposition of agglutinated cells, and with a given highly viscous compound marked differences may exist in the curves obtained with virus and with nitroakridin. Turbidity inherent in the compound or resulting from interaction between compound and red cells or compound and nitroakridin further complicated the issue in some tests. A compound itself agglutinating red blood corpuscles might yet have an inhibitory effect on agglutination due to virus or nitroakridin at the highest or at some of the lower concentrations used. All these and other points were observed and considered, and here we shall do no more than briefly summarize under various headings our interpretation of the results. In general, when we had sufficient compound for replicate tests, the results were reproducible on different days and with different samples of blood. Table I presents examples of typical results obtained with compounds of the various types; these results may be considered in the following categories.

1. At certain concentrations, most of 20 polysaccharides of human or animal origin partly or wholly inhibited agglutination due to living influenza virus. In the Hirst test the unneutralized solution of No. 10 gave readings which were partly vitiated by changes occurring in the red cells. With No. 8 it is uncertain how far the apparent inhibition with virus really represented delayed settling of the red cells in a highly viscous solution; this question receives further attention below. When we had sufficient compound to allow of tests with both influenza viruses, there was usually little difference in the results, but No. 5 and 6 appeared to inhibit agglutination due to influenza A more strongly than that due to influenza B.

With No. 3 to 7 and 11 the inhibitory effect was enhanced when fully-living virus was replaced by virus heated at 56° C. The enhancement was shown by more complete inhibition of agglutination, by an effect over an increased range of dilutions of compound, or by both. This effect was usually greater in the presence of influenza B, but with No. 3, and probably with No. 5 and 6, no difference existed between the two viruses. It will be noted that all the preparations thus affected were of substances related to the specific blood group polysaccharides, which might be expected to behave as "Francis inhibitors." [Since this work was done we have examined a specimen of commercial mucin (The Wilson Laboratories, Chicago) completely inactive against unheated or heated

influenza B or unheated influenza A, but highly inhibitory to heated influenza A. Substances related to the group under consideration, therefore, vary greatly in respect to the virus against which they show maximal activity].

Most of the polysaccharides of the present group also inhibited agglutination due to nitroakridin. Although with such very different agglutinating agents as virus and nitroakridin it may not be legitimate to force comparisons, it is a fact that the results with the two did not run wholly parallel. The effects with No. 4 and 5 and nitroakridin were less marked than the maximal effect obtained with virus. On the other hand, the effect with nitroakridin was often apparently greater, and in one or two cases (e.g. No. 16) in which we could safely use more than 1 HU nitroakridin, the greater effect still persisted with as much as 4 HU. The very marked effect with No. 8, which as stated above forms a highly viscous solution, suggests that viscosity may have been less important in contributing to the results with virus than we imagined, for certainly solutions containing as little as 2 μg./c.c. are not highly viscous.

In further tests compounds 5 and 11 were incubated overnight at 37° C. with 4 HU influenza A virus. Next morning the mixtures were heated for one hour at 56° C. They were then tested for an inhibitory action in agglutination tests with heated virus and with nitroakridin. The inhibitory action with virus was virtually abolished, while the effect with nitroakridin was unchanged. When compound 11 was incubated overnight with influenza B virus, its inhibitory action on viral haemagglutination was completely lost, while its action against nitroakridin persisted. Under the same circumstances compound 5 lost in addition some of its power to inhibit agglutination by nitroakridin.

On several occasions we sought to determine whether red cells deprived of their receptors for the influenza virus were still agglutinated by nitroakridin. After incubation overnight at 37° C. with influenza virus A or B, however, the cells agglutinated spontaneously in saline and the test could not be carried out.

Other substances of human or animal origin tested but not included in Table I were as follows: Blood-groups A and B polysaccharides (porcine and human, respectively); polysaccharides from gastric mucosa (hog), liver (bovine, rabbit, guinea-pig) and lung (bovine); follicle-stimulating mucoprotein; and polysaccharides from commercial trypsin and pepsin.

2. A group of compounds originating from micro-organisms or seaweed (No. 21 to 35) in many cases gave results difficult to interpret. Many of the "solutions" were turbid or viscid, or both, or themselves agglutinated red cells. Thus, with No. 28 the results in the Hurst test could be explained by a failure of agglutinated cells to sediment at the higher concentrations of polysaccharide (but see under 4 below), and when the cells did finally settle-out in the Salk test they were fully agglutinated; nevertheless, the greater apparent effect with nitroakridin is difficult to explain, since this substance agglutinates red cells into coarse clumps which should settle readily, while the solutions of polysaccharide containing as little as 1 or 4 μg. are not highly viscid. The result in the Hirst test with No. 23 was influenced, no doubt, by the turbidity of the polysaccharide and the changes occurring at higher concentrations in the red cells.

Some of the results, however, appeared beyond doubt and were reproducible on several occasions. With No. 21, 22, 24, 32 and 35 agglutination was partially or wholly inhibited, apparently more often when due to nitroakridin than when caused by virus. The effect with nitroakridin was not necessarily seen only

with 1 HU of the chemical; thus with No. 22 it persisted when 4 HU were used. No. 30 partly inhibited agglutination due to virus, but had no effect on that caused by nitroakridin. In no case was there any appreciable difference when virus heated at 56° C. replaced fully-living virus.

Compounds in this group excluded from Table I comprised polysaccharides from *Myco. tuberculosis* and *Penicillium notatum*, a dextran from *Leuconostoc mesenteroides*, levans from *B. megatherium* and *Strept. salivarius*, and a mannan from yeast.

3. A group of relatively simple carbohydrates and related substances (No. 36 to 52) are omitted from Table I. Many of them were strongly acid, changing or destroying the red cells and giving in the Hirst test readings which reflected this change rather than inhibited agglutination; in the Salk test there was no evidence of such inhibition. These compounds, which may be regarded as components of the various polysaccharides considered above, included D-glucuronic, D-galacturonic, lactic, citric, tartaric, saccharic and mucic acids, D-glucurone and acetylated D-glucurone. After neutralization the solutions had no action in either the Hirst or the Salk test. Other compounds—glucosamine hydrochloride, methyl glucuronate, menthyl glucuronate, ammonium menthyl glucuronide, monoacetone D-glucurone, glucose-l-phosphate, hexose di-phosphate and gluconic acid—were without activity.

4. Of special interest was a small group (No. 53 to 59) of what we call "semi-synthetic" compounds, since they were derived by chemical modification of naturally-occurring products. With the possible exception of No. 53, none had any effect on the agglutination of red cells by either influenza virus. In viscosity their solutions ranged from one extreme to the other, yet in the Hirst test the readings at all concentrations of all compounds but No. 53 (not the most viscous) were those of fully agglutinated corpuscles settling down in normal time. This observation suggests that in some cases previously considered where very viscous solutions gave the readings of apparent inhibition, viscosity need not be considered as an important factor if other evidence of inhibition (e.g. a modified pattern in the Salk test) were present. In contrast to their inactivity against agglutination caused by virus the present group of compounds all inhibited agglutination due to nitroakridin, some very strongly.

The compounds of this class not listed in Table I were a sodium carboxymethyl ether of amylose (B.V. 0·007, ratio 1–1·3), a sodium carboxymethyl ether of starch (B.V. 0·420, ratio 1–11·8) and a similar derivative of starch (ratio approximately 1–10).

5. This group comprised a number of synthetic, water-soluble polymers (No. 60 to 68). (We are indebted to Dr. W. A. Sexton and Dr. G. Swain for suggesting and providing these compounds.) One of these, No. 60, partly inhibited the agglutination of red cells by influenza virus A; the result was the same whether living or heat-inactivated virus was used. This polymer also completely inhibited agglutination by nitroakridin (1 and 2 HU), as did also No. 65 and 68. With nitroakridin, No. 63 gave readings characteristic of marked inhibition in the Hirst test, but no inhibition was seen in the Salk test. These results were confirmed on several occasions.

Two samples of (partially hydrolysed) polyvinyl alcohol and one each of ammonium polymethacrylate, polyacrylamide and polymethacrylamide were without action.

6. Compounds 69 to 77 were proteins or their derivatives. Desoxyribonucleic acid (No. 69) partly inhibited agglutination due to nitroakridin. Human globulin (No. 71) inhibited agglutination caused by the influenza viruses, a fact no doubt partly explained by the presence of neutralizing antibodies; this explanation, however, will not serve in the case of similar, though less marked, inhibition of nitroakridin. The other substances examined included ribonucleic acid, thymus histone sulphate, sturgein sulphate, clupein sulphate, gluten, casein and gelatin; they did not inhibit agglutination due to virus and were not tested against nitroakridin. Several of these compounds themselves agglutinated red cells.

In addition to the detailed series of tests with virus and nitroakridin, we examined 14 compounds for an inhibitory action on the haemagglutinin of *Phaseolus vulgaris* (Li and Osgood, 1949). We made and tested two samples of bean-extract, which in our hands caused a very fine agglutination of red cells. The small masses settled slowly, so that after one hour the Hirst readings were those of unagglutinated cells; later, however, when the cells had settled, they were seen to be agglutinated. In the presence of this haemagglutinin no compound inhibited agglutination, while several increased the coarseness of the agglutination and diminished the time of sedimentation of the red cells.

Comment.

After the several complexities and contradictions of these tests have been taken into consideration, it is clear that many of the " natural " polysaccharides partly or wholly inhibited haemagglutination due to influenza virus or to nitroakridin, or both. The effect on viral agglutination was relatively more frequent with products originating from man or animals than from the lower organisms. Only once did a compound (a bacterial dextran) possess activity against virus when devoid of activity against nitroakridin. The reverse situation occurred more commonly. Thus, of the water-soluble polymers, one partly inhibited agglutination due to virus and several that caused by nitroakridin. All the " semi-synthetic " polysaccharides and desoxyribonucleic acid had some effect on agglutination resulting from nitroakridin, but not on that caused by virus. Simpler carbohydrates and related substances were without effect on either. No compound inhibited the action of the agglutinin in extracts of *Phaseolus vulgaris*.

Unless it be found that nitroakridin acts on red cells in a manner similar to influenza virus, these observations suggest that the inhibition of agglutination may indicate no more than a general " protective " effect of certain large molecules on the red cells. In the case of some of the polysaccharides related to the blood group specific substances, evidence of a more specific action may be furnished by the demonstration that influenza virus is able to alter the polysaccharides, so that they no longer act as inhibitors of viral haemagglutination. This is seen in the comparison of the relative effects of heat-inactivated and fully-living virus, a phenomenon described by Francis (1947). Presumably, in these instances, the polysaccharide is identical with or closely related to a cellular constituent (receptor) which the virus is known to destroy during the period in which it is attached to the red cell; here, it seems reasonable to suppose that the inhibition of agglutination may be a specific phenomenon resulting from competition for the virus between free polysaccharide and the cell-receptors. In support, however, of our caution in assessing the significance of inhibition of viral haemagglutination

on other occasions, is the observation that these particular polysaccharides, robbed of their specific properties by incubating with living virus, may still be highly active in inhibiting agglutination by nitroakridin. By analogy it would seem certain that macromolecules exist which can exercise a general " protective " action against agglutination of red cells by virus, and no doubt some of the reactions described in this paper have only this significance.

EXAMINATION OF VARIOUS MACROMOLECULAR SUBSTANCES FOR ABILITY TO MODIFY INFECTIONS IN MICE DUE TO INFLUENZA VIRUS A.

Since, as we have seen, many macromolecular substances interfere with haemagglutination by virus or by nitroakridin, we tested a number for ability to influence the action of influenza virus *in vivo*.

Results.

In from 1 to 3 " 7-day " tests with each of the following compounds, we did not succeed in influencing significantly the mean pulmonary score : No. 3 to 6, 10, 11, 16, 21, 24, 32, 35, 69. The identity of these compounds can be established from Table I. In 5 tests with No. 7 we obtained what was apparently a just significant reduction in the score ($P = 0{\cdot}5$), and in 4 tests with No. 71 very highly significant reduction due, no doubt, to the presence of antibodies for the virus. In single survival tests, compounds No. 28, 53, 72 to 74 and 77 failed to reduce pulmonary lesions or to prolong survival. The last four compounds are respectively histone, sturgein, clupein and gelatin.

Initially encouraging results followed the use of one polysaccharide and a number of water-soluble polymers. In from 5 to 14 tests with each of these compounds, the mean pulmonary scores on the 7th day were almost consistently lower than in control groups ; in the aggregate the results were clearly significant (Table II). When, however, in from 3 to 10 parallel tests with each compound the mean period of survival was taken as the measure of activity, in but one instance (No. 61) did the overall effect just attain significance. In the various tests the concentration of each polymer ranged from 5 to 0·1 per cent, with about the same average effect in reducing lesions. At different concentrations the solutions varied greatly in viscosity, and among themselves the polymers also differed intrinsically in this respect ; no correlation appeared to exist between viscosity and biological effect. On introduction into the respiratory tract, it might be expected that polymer would not quickly leave the surface, whereas the water in which it is dissolved would diffuse into the lymph or blood ; probably all the concentrations used contained sufficient polymer to provide at least a monomolecular layer over the whole respiratory surface. One per cent solutions of three of the polymers (61, 62, 64) and the Type II pneumococcal polysaccharide did not inactivate virus *in vitro*, when held in contact for 5 hours at room temperature and overnight in the ice box. Finally, none of these substances had any action when the infecting dose of virus exceeded 10^{-5} (giving in different control groups a mean pulmonary score of 3·1–4·6).

Table III shows the results of similar tests performed with four " semi-synthetic " polysaccharides and two proteins, all in 1 per cent solution except No. 55, which because of its viscosity could not conveniently be administered at a concentration greater than 0·25 per cent. On these occasions virus was

used at dilutions of 10^{-3}, 10^{-5} and 10^{-6}. Compared with the synthetic polymers, these compounds had less effect in reducing the mean pulmonary scores, but often the mean periods of survival were significantly prolonged by lower mortality among the treated mice. Gluten and compound No. 56 appeared most active in this respect, and were the only ones to show an appreciable effect, albeit inconstantly, with the largest dose of virus. The overall favourable effects with

TABLE II.—*The Effect of Some Macromolecular Substances on Influenza in Mice.*

Number and identity of compound.	7-day tests.		Survival tests.		
	Number of tests.	Mean pulmonary scores.	Number of tests.	Mean pulmonary scores.	Mean periods of survival.
22. Pneumococcus type II—polysaccharide	5	−0·57 (0·37)	3	−0·44 (0·52)	+0·10 (2·28)
60. Polyvinyl alcohol—high viscosity	5	−0·40 (0·35)	..	..	..
61. Polyvinyl alcohol—medium viscosity	14	−0·72 (0·21)	10	−0·64 (0·28)	+1·23 (1·10)
62. Polyvinyl alcohol—low viscosity	14	−0·58 (0·21)	10	−0·50 (0·28)	+0·34 (1·10)
64. Ammonium polymethacrylate	13	−0·52 (0·22)	9	−0·12 (0·32)	−0·43 (1·18)
65. Acrylamide-methacrylic acid	7	−0·70 (0·24)	5	−0·85 (0·32)	+0·85 (1·25)
66. Polyacrylamide	5	−0·78 (0·30)	3	−0·68 (0·39)	+1·45 (1·58)
67. Poly-methacrylamide	5	−0·86 (0·27)	3	−0·37 (0·45)	−0·55 (1·82)
68. Poly-methacrylic acid	5	−0·43 (0·29)	3	−0·65 (0·40)	−0·44 (1·59)

The figures show the difference between the mean pulmonary scores or mean period of survival in animals receiving polymer, etc., prior to virus and those receiving water. They are derived from the grand means of all the tests with a particular compound and two dilutions of virus (10^{-5} and 10^{-6}; the figures in brackets are those needed for significance ($P = 0·05$). The scores in control groups ranged from 3·1 to 4·6 (mean 3·6) for the 10^{-5} dilution of virus and from 2·0 to 3·5 (mean 2·5) for the 10^{-6} dilution. The methods of scoring and of carrying out the "7-day" and "survival" tests are described in the text.

TABLE III.—*The Effect of Some Macromolecular Substances on Influenza in Mice.*

Number and identity of compound.	Number of tests.	Mean pulmonary scores.	Mean periods of survival.
54. Sodium carboxymethyl ether of amylopectin	7	−0·37 (0·25)	+1·36 (0·95)
55. Sodium carboxymethyl ether of starch	6	−0·20 (0·28)	+0·51 (1·05)
56. Sodium carboxymethyl ether of amylose	8	−0·35 (0·23)	+1·72 (0·86)
57. Sodium carboxymethyl ether of starch	8	−0·20 (0·23)	+0·66 (0·86)
75. Gluten	10	−0·43 (0·20)	+1·67 (0·75)
76. Casein	8	−0·16 (0·23)	+1·00 (0·86)

The figures have the same significance as those in Table II. Each compound was tested against three dilutions of virus (10^{-3}, 10^{-5}, 10^{-6}). The mean pulmonary scores in control groups ranged from 4·9 to 5·9 (mean 5·3) for the 10^{-3} dilution, from 4·1 to 5·3 (mean 4·5) for the 10^{-5} dilution, and from 3·3 to 4·3 (mean 3·6) for the 10^{-6} dilution.

other compounds derived largely from the results with the 10^{-5} and 10^{-6} dilutions. By repeating the intranasal instillation of compound 24 hours, or 24 and 48 hours, after infection, we did not obtain more favourable results. The effectiveness or otherwise of the compounds bore no apparent relation to the viscosity of their solutions.

With all the compounds listed in Tables II and III, the results of different experiments varied considerably. Sometimes little or no effect on survival was

seen, and occasionally the mean period of survival was shorter than in controls receiving a preliminary instillation of water. On the other hand, in some experiments significantly increased mean periods of survival occurred in all groups of treated mice, due to the survival of many individual animals. The variation between experiments was much greater than in replicate tests performed over the same period by the same operators carrying out routine chemotherapeutic experiments with influenza.

Comment.

It seems, therefore, that several macromolecular substances of very dissimilar chemical structure may, when administered intranasally to mice four hours before infection with influenza, protect some individuals from a fatal outcome and hence increase the mean period of survival. Most of these substances do not inhibit agglutination of red cells by virus. Their chemical diversity and the fact that if they are administered after as well as before virus no more favourable results accrue, suggest perhaps that they act mechanically by preventing virus from reaching as large a number of susceptible cells as in controls treated merely with water.

SUMMARY.

None of over 160 bacterial cultures instilled intranasally into mice 4 hours before influenza A virus (PR8 strain) protected the animals against infection.

Many polysaccharides of animal and vegetable origin and one synthetic water-soluble polymer interfered with agglutination of fowl red cells by influenza A or B virus. A larger number inhibited agglutination caused by Nitroakridin 3582. Only one compound (a dextran from *Betacoccus* Birmingham) partly inhibited agglutination by virus while having no effect on that caused by nitroakridin. None of 14 compounds tested inhibited the haemagglutinin of *Phaseolus vulgaris*.

In most instances there was nothing to suggest that these effects were due to anything but a general " protective " action of the macromolecules on the red cells. With some of the polysaccharides of animal origin, however, a more specific interference was suggested by the fact that unheated influenza virus could so modify the polysaccharide that it no longer inhibited viral agglutination, but might still inhibit that due to nitroakridin.

The intranasal instillation four hours before virus of some macromolecular substances, of which gluten and a sodium carboxymethyl ether of amylose were the most active, prevented some mice from acquiring a fatal influenzal infection. The evidence suggested that their action was mechanical rather than truly therapeutic.

We are indebted to Dr. O. L. Davies for statistical analysis of our results.

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APPENDIX: ORIGIN OF THE POLYSACCHARIDES, ETC., USED IN THIS INVESTIGATION.

These were samples of material already described in the following references or prepared as briefly indicated. With such complex molecules, no claim can be made that they were completely homogeneous. All of the samples made from human or animal tissues probably contained blood-group polysaccharides. The numbers correspond with those in Table I.

3. W. T. J. Morgan and M. B. R. Waddell. *Brit. J. exp. Path.* (1945), **26**, 387.

4. Serum-mucoid—a human sample—received from Professor J. T. Edsall (private communication).

5. Ovarian-cyst fluid (520 c.c.) received through the kindness of Professor Haswell Wilson, was diluted with water (200 c.c.) and then boiled for 3 minutes after addition of potassium carbonate (2 g.). The solution was acidified by addition of acetic acid. It was then deproteinized by the method of M. G. Sevag (*Biochem. Z.*, 1934, **273**, 419). The solution was evaporated under diminished pressure to 50 c.c., centrifuged and then poured into ethanol (5 vol.). The product isolated on the centrifuge was purified by dialysis and solvent fractionation methods. The potassium salt of an acidic fraction, K : 12·2 per cent $[\alpha]_D^{20}$—40° (c. 1·1 in water), N : 5·7 per cent was used.

6. The method used was essentially the same as that described for compound 5. The fluid received from Professor Haswell Wilson was described as being from a malignant cyst. The sample used was a potassium salt, K : 9·0 per cent $[a]_D^{20}$—22·0° (c. 1·2 in water), N : 4·8 per cent.

7. Prepared by degradation of sample 8 by heating with potassium carbonate.

8. J. Madinaveitia and M. Stacey. *Biochem. J.* (1944), **38**, 413.

10. M. Stacey and J. M. Woolley. *J. chem. Soc.* (1942), 550.

11. The liver (200 g.) was minced and covered for a few days with a solution of phenol (1 per cent) until all glycogen had disappeared and some autolysis had taken place. The mass was boiled for 18 hours with five times its weight of water. After filtration through cloth the extracts were evaporated under diminished pressure to 200 c.c. and the solution deproteinized by the chloroform-butanol method. The residual solution was evaporated to 10 c.c. and polysaccharide material in solution precipitated with 3 volumes of acetone. It was purified by fractional precipitation methods with solvents. The product (2·5 g.)—a white powder—was protein-free $[\alpha]_D^{20}$ + 15°, contained bound phosphate (P : 1·8 per cent), galactose residues and traces of an amino sugar. The product was not further characterized.

16. Finely powdered dried sheep pituitary (300 g.) was extracted with boiling acetone (10 vol.) and then with very dilute sodium carbonate solution (1 litre) at 70° for 3 hours. The filtered solution was evaporated to 50 c.c. and this solution deproteinized by addition of trichloroacetic acid until no further precipitate was thrown down. After removal of the precipitate on the centrifuge, the solution was poured into alcohol giving a polysaccharide in the form of a white powder. It was purified by dialysis and solvent fractionation and the polysaccharide used was a protein-free product $[\alpha]_D^{20}$—30° (c, 1·5 in water), N : 5·5 per cent. It consisted mainly of a galactan readily hydrolysed by $\frac{N}{10}$ mineral acid to D-galactose and an amino sugar.

21–23. B. R. Record and M. Stacey, *J. chem. Soc.*, (1948) 1561.

24. M. Heidelberger and D. G. Anderson. *J. clin. Invest.* (1944), **23**, 607, and private communication from M. Heidelberger.

28. E. A. Cooper, W. D. Daker and M. Stacey. *Biochem. J.* (1938), **32**, 1752.

30. M. Stacey and G. Swift. *J. chem. Soc.* (1948), 1555.

32. C. G. Anderson, W. N. Haworth, H. Raistrick and M. Stacey. *Biochem. J.* (1939), **33**, 272.

35. This sample was a reprecipitated sample of the commercial alginic acid " manucol."

53. This sodium salt of the carboxymethyl ether of cellulose was prepared by Imperial Chemical Industries Limited, Ardeer.

54. This " 1–10 derivative " of amylopectin was prepared by the method described by E. J. Bourne, S. Peat and R. D. Thrower (*Nature*, 1947, **159**, 810). The ratio signifies the proportion of carbomethoxy groups to the glucose residues.

55. The preparation of higher substituted derivatives requires an excess of the sodium monochloracetate. The method used for preparing the sodium salt of the carboxymethyl ether of starch (ratio 1 : 2) described below, gives the quantities and method as used for higher substituted derivatives with the approximate ratio of 1 : 1.

Starch (50 g.) was creamed with water (100 c.c.), added slowly to vigorously stirred boiling water (400 c.c.), and the suspension allowed to boil for 1 hour with stirring. To the cold suspension sodium hydroxide solution (300 c.c. of 50 per cent) was added, and the alkaline solution stirred whilst the temperature was raised to 55° C. A solution of sodium monochloracetate (prepared by neutralizing 500 g. monochloracetic acid with caustic soda and making up to 500 c.c.) also at 55° C. was slowly added over a period of 1 hour. The temperature was maintained at 55° C. with stirring for 3 hours. The reaction mixture was then kept at room temperature for 18 hours and neutralized with glacial acetic acid. Electrolytes were removed by dialyzing against running water for 3½ days. Precipitation of the carboxymethyl ether was by the addition of alcohol, the product thus obtained being ground under alcohol until it was transformed into a powder. Further impurities were removed by extraction with 92 per cent alcohol in a soxhlet apparatus for 6 hours, the final product being ground under alcohol again to harden it. Yield 39 g.

59. Prepared by the oxidation of cellulose with N_2O_4. M. Heidelberger and G. L. Hobby. *Proc. nat. Acad. Sci., Wash.* (1942), **28**, 516.

THE INFLUENCE OF CHEMICAL CONSTITUTION ON ANTIBACTERIAL ACTIVITY. PART V: THE ANTIBACTERIAL ACTION OF 8-HYDROXYQUINOLINE (OXINE).

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In Part III of this series (Albert, Rubbo, Goldacre and Balfour, 1947) 8-hydroxyquinoline and its isomerides were examined for antibacterial activity. Of the seven isomeric mono-hydroxyquinolines, only oxine (8-hydroxyquinoline) was active, and only oxine was found to be capable of forming non-ionized complexes with divalent metallic ions, a process known as chelation (Albert and Gledhill, 1947). On the evidence then available it was thought that this undoubted connection between chelation and antibacterial activity might be due to the masking (or removal) by oxine of a metallic ion forming an essential part of a metabolic process.

The present study is an extension of Part III, in which a closer analysis of the mode of action of oxine has been made using a greater variety of test organisms. As a result of further work it has become possible to confirm our original conception that oxine acts by chelating with the ions of certain heavy metals. The mechanism by which this chelation injures the cell is found, in the case of Gram-positive organisms at least, to be more complex than had been suggested. Sufficient data have now been accumulated to show that the mode of action of oxine is distinct from that established for other antibacterial agents.

The observations recorded here show that oxine is active against staphylococci and other Gram-positive types only when iron or cupric ions are present in the medium. Oxine is devoid of all antibacterial activity in media where the concentration of these ions falls below a necessary, though truly minute, value. These observations are open to two interpretations. The first is that oxine reacts with the iron or cupric ion to form a toxic metal-oxine complex which exerts a direct lethal action. The second is that oxine removes a metallic component from a vital cellular enzyme-system, thus exposing an oxidizable group (normally protected by the metal) to destruction by an iron- or copper-catalysed oxidation. It will be evident from this paper that any complete explanation of the mode of action of oxine must account for a number of apparently unrelated phenomena.

EXPERIMENTAL.

The oxine used in this study was obtained from Hopkin & Williams, Ltd., as white elongated crystals, m.p. 74° C.

Spectrographic investigation of media and bacteria for trace metals was carried out by Mr. A. C. Oertel of the Waite Institute, Adelaide, and by Mr. A. Sinclair of the Defence Research Laboratories, Melbourne.

The treatment of broth media to remove heavy metals was similar to that

described by Waring and Werkman (1942), but with the following modifications: Instead of relying on three treatments of the medium with oxine (and subsequent immediate chloroform extraction of the oxine-metal complexes), we found it necessary to use, on an average, ten to twelve treatments with oxine. Furthermore, after each treatment the reagent was not extracted with chloroform until several hours had elapsed. Lastly, no less than fourteen chloroform washings of the broth were finally required to remove the last traces of oxine. This somewhat cumbersome procedure was found to be necessary because of the complexity of the media, viz. (i) broth containing beef extract (0·5 per cent) and

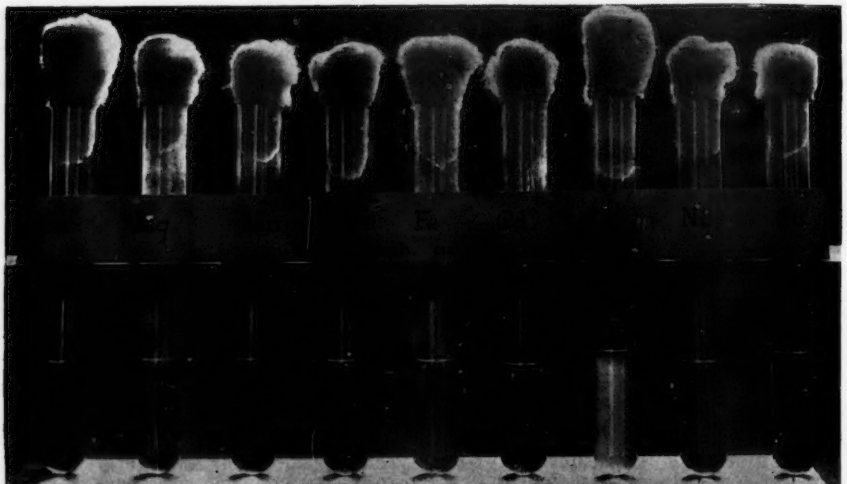

FIG. 1.—Unique action of cobaltous ion in antagonizing the antibacterial action of oxine.

Difco peptone (1 per cent), and (ii) brain heart infusion broth (Difco). The final pH was 7·3 to 7·4. The broth was sterilized, dispensed and inoculated as described in Part III.

When using oxine-treated media, or twice glass-distilled water, stringent precautions were necessary to exclude trace metals from contaminating reagents and apparatus. For example, all glassware, usually Pyrex, was thoroughly rinsed in glass-distilled water, then 2 per cent alcoholic potassium hydroxide, followed by distilled water, then aqua regia (diluted 1 in 3), and finally rinsed six times with double distilled water, as described by Waring and Werkman (1942). Each batch of distilled water was tested with dithizone to guard against accidental contamination with heavy metals. Cotton-wool plugs, when used, were always covered with dust-free cellophane.

RESULTS.

1. *Tests for antagonism of oxine activity by metals.*

These tests were designed to show by a simple technique whether any metal or metals would protect organisms from the inhibitory action of oxine in broth. The concentration of oxine selected in each case was the lowest found to inhibit the growth of the test organism after 48 hours' incubation. Metal solutions were added in four times the theoretical equivalent required to combine with the oxine present. The metallic salts used, of Analytical Reagent quality, were calcium and ferric chlorides, manganese, cadmium, ferrous, cobalt, zinc, nickel and copper sulphates. Controls containing the same metallic concentration but no oxine, and controls with oxine but no added metallic salt, were included in each experiment. A series of tubes so prepared was inoculated with 0·03 ml. from an 18- to 24-hour culture of the test organism. The tubes were then incubated for 48 hours at 37° C. and the degree of growth recorded.

The main results, which are summarized in Table I, will be more readily comprehended after reference to the following protocol, which is an extract from the Table (in a less condensed form). Fig. 1 relates to the same data.
Organism : Staph. aureus.

Broth contains—	Growth in tubes containing Ca++.	Mg++.	Mn++.	Cd++.	Fe++.	Co++.	Zn++.	Ni++.	Cu++.	No added metal.
Metallic salt (M/50,000)	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Metallic salt (M/50,000) + oxine (M/100,000)	—	—	—	—	—	+++	—	—	—	—

+++ Signifies maximum growth. — Signifies no growth.

Interpretation : Strong antagonism of oxine by cobalt. It should be noted that no metal was too toxic to allow this test to be performed.

It will be seen here that the oxine concentration was inhibitory to the organism, and that this inhibition was unaffected by the presence of eight of the nine added metallic ions (which are listed in order of their increasing affinity for oxine at all pH values). However, in the presence of cobaltous ions the organisms grew profusely ; this was interpreted as a strong antagonism of oxine action by cobalt. It may be added here that an antagonism of this type is demonstrable only by the simultaneous addition of the inhibitor (oxine) and the antagonist (cobalt) to the culture medium because oxine is so rapidly bactericidal. Control tubes containing the inhibitory concentration of oxine, without added cobalt, were found to be sterile after 1 to 2 hours.

In Table I we have listed the results of similar experiments with 21 different species of micro-organisms using 9 different metals. These tests have been repeated many times and give reproducible results for oxine-sensitive organisms, namely, those which are inhibited by a concentration of M/25,000 or less. When higher concentrations of oxine were required for inhibition, as in the case of

TABLE I.—*Antagonism of Oxine Action in the Presence of Divalent Metals.*

Medium: Beef extract, peptone water. *Incubation time:* 48 hours at 37° C.
Metals used: Ca, Mg, Mn, Zn, Fe^{++}, Cd, Co, Ni, Cu.

No.	Organisms.	Medium contains— Oxine 1/M.	Medium contains— Metal 1/M.	Metals which reverse (indicated by degree of growth). Good +++, ++.	Metals which reverse (indicated by degree of growth). Slight +, ±.	Metals too toxic to test at the 4 equiv. concentration used.
	Gram-negative rods :—					
1.	*Ps. pyocyanea*	400	200	Zn, Fe	Mn	Co, Ni, Cu
2.	*Proteus* sp.	800	400	Zn, Fe	..	Co, Cu
3.	*Bact. coli*	1,600	800	Zn, Fe, Cu	Ni, Mn	..
4.	*Bact. aerogenes*	3,200	1,600	Zn, Fe, Cu	Co, Ni	Cd
5.	*Salm. typhi*	3,200	1,600	Zn, Fe	Co, Cu	Cd
6.	*Sh. schmitzi*	3,200	1,600	Fe, Cu	Zn, Ni	Cd
7.	*Past. pseudotuberculosis*	6,400	3,200	Fe, Cu	Zn, Ni	Cd
8.	*Sh. flexneri* Type IV	12,500	6,400	Fe	Co, Cu	Cd
9.	*Br. abortus*	25,000	12,500	Fe, Co	..	Cd
	Gram-negative Diplococci :—					
10.	*N. catarrhalis*	50,000	25,000	Co	..	..
11.	**N. meningitidis*	100,000	50,000	Co	Fe	Cd
12.	**N. gonorrhoeae*	200,000	100,000	Co	Cd	..
	Gram-positive Species :—					
13.	*Strept. lactis*	12,500	6,400	Co	..	Cd
14.	†*Strept. pyogenes* (4 different strains)	100,000	50,000	Co	Fe	Cd
15.	†*Haem. streptococcus* (Group B)	100,000	50,000	Co	..	..
16.	†*Haem. streptococcus* (Group C)	50,000	25,000	Co	..	Cd
17.	**Strept. viridans*	100,000	50,000	Co	..	..
18.	*Staph. aureus* (2 different strains)	100,000	50,000	Co	..	..
19.	*Staph. aureus* (3 penicillin-fast strains)	100,000	50,000	Co	..	..
20.	**C. diphtheriae mitis*	100,000	50,000	Fe, Co	..	..
21.	*B. subtilis*	25,000	12,500	Co	..	Cd

* 10 per cent serum added. † 0·1 per cent glucose added.

most Gram-negative bacilli, the metal concentrations had to be increased accordingly and this often resulted in coloured precipitates which greatly interfered with the reading and interpretation of results. However, this variability does not affect the main subject of this paper, viz. the mode of action of oxine on the highly sensitive Gram-positive cocci.

The most striking feature in Table I is the almost specific antagonism by cobalt of the action of oxine on all Gram-positive bacteria and on the *Neisseria*. On the other hand, no single ion is specific for the most resistant of the Gram-negative bacilli where zinc, iron and copper are the most successful antagonists, and nickel, cobalt and manganese are somewhat effective for a lesser number of species. Thus, no less than 5 of the 9 ions antagonize the action of oxine on *Bact. coli*. However, as the sensitivity of the organism increases, the multi-metal nature of the antagonism recedes and, in the case of *Br. abortus*, it closely approximates to the type seen with the Gram-positive organisms.

2. The antagonism by cobalt.

The antagonism of oxine by cobalt, as shown in Table I, merely indicates that the addition of cobalt to a medium permits the growth of Gram-positive organisms in what would normally be an inhibitory concentration of oxine. The following experiments provide information of a more quantitative nature.

Table II shows a titration with *Staph. aureus* and *Strept. pyogenes* in which the cobalt concentration was maintained at 4 equivalents for each of the varying oxine concentrations. It will be seen that an eight-fold antagonism of oxine activity was obtained with *Strept. pyogenes* and a sixteen-fold with *Staph. aureus*. Hence antagonism by cobalt is not complete, and inhibition of growth still operates at M/6400, in spite of the presence of an excess of cobalt. An explanation will be offered later.

TABLE II.—*Antagonism of Oxine Action by 4 Equivalents of Cobaltous Sulphate.**

Medium: Beef extract, peptone water, pH 7·3. *Incubation time:* 48 hours at 37° C.

Organism.	Medium contains—	Concentration of oxine 1/M. 6,400	12,500	25,000	50,000	100,000	200,000	400,000	Comment.
Staph. aureus†	No added cobalt	—	—	—	—	—	++	++	..
	4 equiv. of cobalt added to each dilution	—	+	+	++	++	++	++	16-fold reversal
Strept. pyogenes‡	No added cobalt	—	—	—	—	—	—	++	..
	4 equiv. of cobalt added to each dilution	—	—	—	+	++	++	++	8-fold reversal

* Control tests with cobaltous sulphate ($CoSO_4.7H_2O$) in broth showed no inhibition of growth at M/1600.
† One atom of cobalt in solution is equivalent to two molecules of oxine.
‡ 0·1 per cent glucose added to the medium.

TABLE III.—*Antagonism of Oxine Action by Varying Amounts of Cobaltous Sulphate.*

Medium: Beef extract, peptone water, pH 7·3. *Incubation time:* 48 hours at 37°C.

Organism.	Medium contains—	Concentration of cobaltous sulphate 1/M. 25,000	50,000	100,000	200,000	Control no added cobalt.	Comment.
Staph. aureus	M/100,000† oxine	+++	+	+	—	—	2 equivalents of cobaltous sulphate required for reversal.
	No oxine	+++	+++	+++	+++	+++	
*Strept. pyogenes**	M/100,000 oxine	+++	++	+	—	—	2 equivalents of cobaltous sulphate required for reversal.
	No oxine	+++	+++	+++	+++	+++	

* 0·1 per cent glucose added to the medium.
† M/100,000 oxine is equivalent to M/200,000 of a cobaltous salt.

Next, these experiments were adapted to establishing the minimum amount of cobalt required to antagonize oxine when the concentration of the drug was kept constant. The result, shown in Table III, indicates that 2 theoretical equivalents of cobalt will antagonize the action of oxine, but 1 equivalent will not.

All the above evidence is consistent with the antagonism being due to the formation of a cobalt-oxine complex. Some of the results in Table II suggest that high concentrations (M/6400 and M/25,000 respectively) of this complex can actually inhibit growth of staphylococci and streptococci. This finding raised the question of the relative toxicity and stability of the other metal-oxine complexes. Could the specificity of cobalt as an antagonist (in the case of the Gram-positive and other oxine-sensitive organisms) be due to the relatively low toxicity and high stability of the cobalt-oxine complex ?

3. *Experiments with oxine-treated media.*

In the absence of any information at this stage on the relative stabilities towards dissociation of the metal-oxine complexes, it was decided to try to render our media [beef extract peptone water (Difco) and brain heart infusion broth (Difco)] relatively free of ionized metals by treatment with oxine and subsequent chloroform extraction of the complexes. The technique was based on that used by Waring and Werkman (1942), and has been described above. For convenience, media treated with oxine will be referred to as O.T. media. It was noted (cf. Tables IV and V) that the inhibitory end-point for *Staph. aureus* was consistently higher in beef extract peptone water (M/100,000 oxine) than in brain heart infusion medium (M/25,000). This is attributed to the high concentration of phosphates present in the latter medium (see section 4 of these Results).

The determination of the bacteriostatic action of oxine in media, after varying degrees of extraction with oxine and chloroform, yielded most interesting and unexpected results. Table IV shows that, as the number of treatments with oxine progressed, the bacteriostatic end-point decreased until after 10 treatments oxine could not inhibit the growth of *Staph. aureus* even at a concentration as high as M/400. This represented a reduction in activity greater than 250-fold. It was clearly evident that oxine had removed something from the media essential for the exhibition of its antibacterial activity. The known chemical reactions of oxine indicated that the treatment had removed the ions of one or more metals essential for the antibacterial action of oxine. Chloroform extraction in the absence of oxine did not affect the inhibitory action of the drug. The interesting prozone type of result obtained after 3 extractions will be discussed in a later publication.

TABLE IV.—*Influence of Oxine Treatment of Medium on the Inhibitory Action of Oxine.*

Medium : Beef extract, peptone water, pH 7·3. *Organism :* *Staph. aureus.* *Incubation time :* 48 hours at 37° C.

	Concentration (1/M) of oxine.										Progressive reduction of titre.
	400	800	1,600	3,200	6,400	12,500	25,000	50,000	100,000	200,000	
Untreated medium .	—	—	—	—	—	—	—	—	—	++	*Nil.*
After 3 treatments .	++	++	+	—	—	—	—	—	++	+++	Slight.
„ 6 „ .	—	—	+	±	+	++	++	+++	+++	+++	64-fold.
„ 10 „ .	++	++	+++	+++	+++	+++	+++	+++	+++	+++	>250-fold.

It seemed important to know whether this phenomenon, that is, loss of activity in O.T. media, was peculiar to *Staph. aureus* or could be demonstrated with other species. Accordingly the inhibitory end-points for a number of organisms in O.T. media were determined. The results of these titrations are recorded in Table V.

TABLE V.—*Inhibitory Action of Oxine in Oxine-treated Brain Heart Infusion Medium.**

Incubation time : 48 hours at 37° C.

Organism.	Highest dilution (1/M) of oxine inhibiting visible growth.		Effect on inhibitory titre.
	Untreated medium.	O.T. medium.	
Bact. coli	3,200	1,600	No change.
Sh. flexneri	12,500	12,500	,,
Br. abortus	25,000	25,000	,,
C. diphtheriae mitis	100,000	25,000	Moderate lowering.
B. subtilis	25,000	1,600	Marked lowering.
Staph. aureus	25,000	<800	,, ,,
Strept. pyogenes	100,000	<800	,, ,,

* Similar results were obtained in beef extract peptone medium.

It will be seen that the end-points for the Gram-positive bacteria, *Staph. aureus*, *Strept. pyogenes*, *C. diphtheriae* and *B. subtilis* were all reduced, whereas with the Gram-negative bacilli, *Bact. coli*, *Sh. flexneri* and *Br. abortus* no reduction was noted. This finding suggested that the action of oxine is qualitatively different for Gram-positive and Gram-negative bacteria.

In an attempt to discover what metal or metals were essential for the action of oxine against Gram-positive forms, the following experiments were made. *Staph. aureus* was titrated against oxine in O.T. media to which salts of various divalent metals were added separately at a concentration of M/25,000. The results are shown in Table VI.

TABLE VI.—*Effect of the Addition of Metal Salts to Oxine-Treated Medium.*

Medium : O.T. brain heart infusion, pH 7·3. *Organism : Staph. aureus.*
Incubation time : 48 hours at 37° C.

The metals are arranged in the order of their increasing affinity for oxine.

Medium contains M/25,000 of the following salts :	Highest dilution of oxine inhibiting visible growth.	Comment.
(Control)	800	No potentiation.
Calcium chloride	800	,,
Magnesium sulphate	800	,,
Manganese sulphate	800	,,
Cadmium sulphate	12,500	Potentiation.
Ferrous sulphate (or ferric chloride)	25,000	,,
Cobalt sulphate	800	No potentiation.
Zinc sulphate	800	,,
Nickel sulphate	800	,,
Copper sulphate	200,000	Marked potentiation.
Untreated brain heart infusion (for comparison)	25,000	

The findings (Table VI) were most striking in that they showed a clear-cut example of potentiation of oxine-activity by iron, copper or cadmium ions. These results, read in conjunction with those listed in Table IV, prove that oxine has little or no antibacterial activity unless one of these ions is present in the medium. This finding was further confirmed by demonstrating that oxine had no action in distilled water unless these metals were present (this work will be published later). Furthermore, it was found that the addition of cobaltous sulphate to "iron- or copper-potentiated" O.T. media effectively reversed oxine-inhibition in a manner similar to that previously described in Table II. For example, in O.T. medium (beef extract peptone) containing 4 equivalents of ferrous sulphate (for each concentration of oxine) inhibition of *Staph. aureus* was obtained at M/50,000, but in the presence of 4 equivalents of cobalt sulphate in addition to the ferrous salt, the inhibitory end-point was reduced to M/6400 oxine. In a medium containing copper sulphate, cobalt sulphate was also antagonistic, but to a lesser degree.

In order to gain more quantitative information on the amount of metal required for potentiation, titrations were performed in O.T. broth to which varying amounts of metallic salts were added. The results are shown in Table VII.

TABLE VII.—*Potentiation of Oxine by Iron and Copper Salts.*

Medium : O.T. beef extract, peptone water, pH 7·3. *Organism : Staph. aureus.* *Incubation time :* 48 hours at 37° C.

Salt added.	Concentration. Parts per million (as free metal).	Concentration. 1/M.	Highest dilution of oxine inhibiting visible growth 1/M.
$FeSO_4.7H_2O$	0·3	200,000	50,000
	0·6	100,000	100,000
	1·2	50,000	100,000
$CuSO_4.5H_2O$	0·3	200,000	50,000
	0·6	100,000	200,000
	1·2	50,000	200,000
No metallic salt added	..	..	<800

It will be noted that quite small amounts of iron and copper are sufficient to potentiate the action of oxine, the maximum effect being obtained with as little as approximately 0·6 parts per million of each metal (i.e. 6×10^{15} atoms per ml.).

Repeated passage of the medium through a kation-exchange column (Amberlite I–R 100) failed to effect the removal of sufficient iron and/or copper to lower the antibacterial action of oxine in the effluent medium.

4. *Metal analyses of oxine-treated and untreated media.*

Twenty ml. samples of media and metabolism solutions in acid-washed pyrex flasks were vacuum-dried prior to qualitative spectrometric (spark) analyses for their metal content. The metabolism solutions were cell-free supernatants, obtained by centrifugation, of 24-hour aerated cultures of *Staph. aureus.* The cellular sediments from these cultures were washed twice in double-distilled water and analysed for their metal content after vacuum-drying. The results of the analyses made individually by the two authorities cited above were identical, and failed to reveal any qualitative difference in metal-content of the medium

before and after oxine treatment. This is hardly surprising, because there is no known method for completely removing heavy metals from an organic solution. These results, when considered in the light of our biological findings, proved that the exhaustive oxine treatment of the media partially, but not completely, removed the potentiating metals, iron and copper. Their significance will be dealt with later (see Discussion).

5. *Antagonism by lowering the oxidation-reduction potential of the medium.*

At an early stage in this work it was thought that oxine might exert its anti-bacterial action by exposing to oxidation a thiol (–SH) group, normally protected by a metal covalently bound, e.g. $-SCo^{+}$. Accordingly a number of titrations were carried out under anaerobic conditions, and in the presence of cooked meat and reducing substances such as thioglycollic acid, cysteine hydrochloride and ascorbic acid.

Under anaerobic conditions (anaerobic jar) there was no marked or consistent antagonism of oxine action but, as will be seen in Table VIII, the addition of the above-mentioned reducing substances, including cooked meat, markedly depressed the activity of oxine against oxine-sensitive types, such as *Staph. aureus* and *Strept. pyogenes*.

TABLE VIII.—*Antagonism of Action of Oxine by Lowering of the Oxidation-Reduction Potential of the Medium.*

Highest dilutions of oxine inhibiting growth after 48 hours' incubation at 37° C.

Medium: 0·1 per cent nutrient agar buffered to pH 7·0 containing—	Organism. *Staph. aureus.*	*Strept. pyogenes.*	*Br. abortus.*	*Sh. flexneri.*	*Salm. typhi.*	*Bact. coli.*	*Proteus* sp.
No added substance	25,000	50,000	25,000	12,500	3,200	3,200	800
Glucose, 1 per cent	25,000	50,000	50,000	12,500	3,200	3,200	800
M/400 ascorbic acid	6,500	800	25,000	6,400	3,200	1,600	800
M/400 sodium thioglycollate	1,600	800	6,400	3,200	1,600	800	800
M/400 sodium thiosalicylate	1,600	800	12,500	3,200	1,600	1,600	800
M/400 cysteine hydrochloride	800	800	6,400	3,200	1,600	800	800
Beef extract peptone	100,000	100,000	25,000	3,200	3,200	3,200	800
Beef extract peptone + cooked meat	1,600	1,600	3,200	800	<800	<800	<800

In regard to other organisms shown in Table VIII, it appeared that the effect of lowering redox potential diminished as the sensitivity of the organism decreased. Thus we see a graded effect: streptococci and staphylococci initially sensitive to oxine were insensitive in the presence of thiol compounds, ascorbic acid or cooked meat, whereas the normally resistant organisms (*Proteus*, *Bact. coli* and *Salm. typhi*) were either not affected or only to a minor degree. *Sh. flexneri* and *Br. abortus* of intermediate sensitivity between these extremes showed an intermediate degree of change in sensitivity. As indicated earlier, oxine is less effective against *Staph. aureus* and *Strept. pyogenes* in phosphate-buffered medium than in beef extract peptone medium most probably because important amounts of metallic ions (e.g. iron and copper) are lost by precipitation as phosphates.

On the other hand, potentiometric titrations showed that ascorbic acid has no tendency to remove heavy metallic ions, so that there can be no question of its competing directly with oxine as do the phosphate buffered media. However,

the thiols (sodium thioglycollate, sodium thiosalicylate, cysteine and cooked meat) exert a strong attraction for ferrous ions and, in the amounts present here, it is just conceivable they may deny oxine the minute amount of iron essential for its activity. For example, the logarithmic stability constant of the ferrous-thioglycollate complex was found potentiometrically to be 9·1, whereas that of the ferrous-oxine complex is 15·0. Using the relative ionization constants, it is apparent that oxine has four times as great an affinity for iron as sodium thioglycollate has, but when (as here) there is 125 times as much thioglycollic acid as oxine, the former gets 125/4, or 31 times as much iron as the oxine.

The results obtained with ascorbic acid opened up new avenues for investigation. For example, they suggested that the insensitivity of the Gram-negative

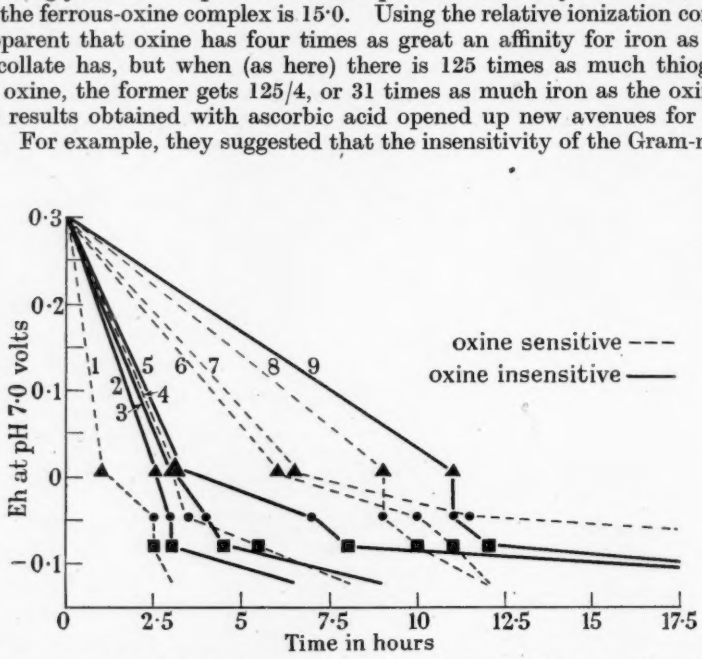

FIG. 2.—Comparison of rates of reduction of oxidation-reduction indicators by different species.

Key 1. *Strept. lactis* in glucose broth.
2. *Bact. coli* in nutrient broth.
3. *Salm. typhi* ,, ,,
4. *Staph. aureus* ,, ,,
5. *Sh. flexneri.* ,, ,,
6. *Strept. lactis* in nutrient broth.
7. *B. subtilis* ,, ,,
8. *Strept. pyogenes* ,, ,,
9. *Ps. pyocyanea* ,, ,,

bacilli was, in part, due to the ability of these organisms to lower the redox potential in their immediate environment before visible growth had occurred. This was investigated by noting the decolorization times of redox indicators during growth of oxine-sensitive and insensitive organisms. Buffered nutrient broth tubes (pH 7·0) containing 0·1 per cent of agar and indicator were inoculated with 0·03 ml. of a 24-hour broth culture of each organism and incubated at 37° C. The tubes were compared at half-hourly intervals with the uninoculated control tubes. The results, which may be of general interest, are shown in Fig. 2.

It will be clear that the *rate* of reduction of redox indicators by the various cultures was in no way correlated with their sensitivity or insensitivity to oxine. In fact, this was even more forcibly demonstrated by titrating *Strept. lactis* in plain broth and in glucose broth against oxine, and at the same time determining

the rate of the decolorization of indicators. In broth the end-point was actually one tube lower (M/25,000) than in the glucose medium (M/50,000) although the time of decolorization of indicators, for instance potassium indigo disulphonate, decreased from 12 hours to 3 hours in the presence of glucose.

Although the *rate* of reduction is evidently not critical, it was thought that the *degree* of reduction finally obtained may be. It became of immediate interest to determine whether there was a critical Eh level at which oxine failed to inhibit growth. In order to do this, a buffered semi-solid medium was prepared, containing oxine in a final concentration of M/13,000. This was dispensed in ten 19-ml. quantities in test-tubes. To four of these tubes, 1 ml. portions of ascorbic acid solutions were added to give concentrations ranging from M/500 to M/32,000, to another four tubes cysteine hydrochloride solutions were added to give the same concentrations, and the remaining two were made up to volume

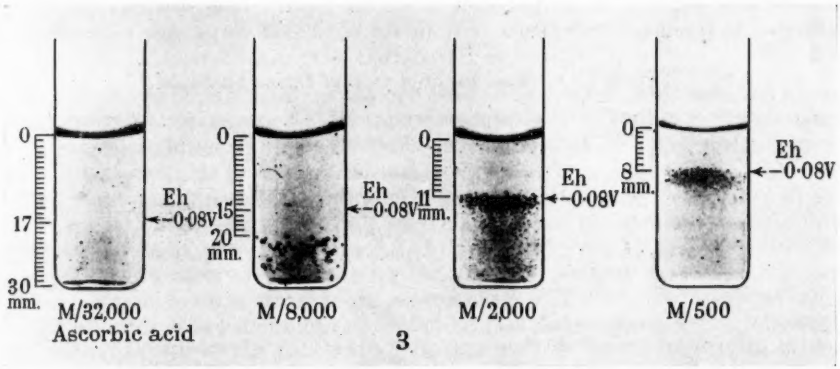

FIG. 3.—Antagonism of action of oxine by lowering of oxidation-reduction potential.
Organism : Streptococcus pyogenes. *Oxine concentration :* M/13,000.
Incubation time : 48 hours at 37° C.

with distilled water to serve as controls. From each of the above tubes five small screw-cap phials of constant length and diameter (50 mm. × 15 mm.) were filled, thus giving five series of tubes, labelled respectively A, B, C, D and E, for ascorbic acid and five, similarly labelled, for cysteine.

To series A one drop of methylene blue solution was added, to B potassium indigo tetrasulphonate, and to C potassium indigo trisulphonate, each at a concentration just sufficient to colour the medium. Series D was inoculated with 0·03 ml. of a 24-hour culture of *Staph. aureus*, and E with a similar inoculum of *Strept. pyogenes*. All tubes were handled in an identical manner, and incubated in a sealed condition for 48 hours at 37° C. The results were read by noting the level at which growth commenced below the surface of the medium, and comparing this with the level at which the decolorization of the indicators occurred in the uninoculated tubes. A typical result is shown in Fig. 3, in which it will be seen that the effectiveness of oxine inhibition progressively decreased as the concentration of the reducing agent (ascorbic acid) increased.

In general a fair correlation was found between the upper level at which growth occurred, that is, the level of antagonism of the inhibitory action, and the level at which the indigo dyes were decolorized in the control media. It was concluded that, at Eh levels below approximately −0·06 volt at pH 7, oxine is relatively inactive against *Staph. aureus*, whereas, in the case of *Strept. pyogenes*, somewhat lower Eh levels (− 0·08 volt) appear to be necessary for antagonizing the action of oxine. These findings indicate why it is that anaerobic cultivation alone is not sufficient to antagonize the antibacterial effect.

6. *Effect of blood on oxine activity.*

So far we have been concerned with the antagonism of oxine activity by metals (particularly cobalt), by the lowering of redox potential, and (indirectly) by phosphates. During the course of the investigation a fourth antagonist was discovered, namely blood. As will be seen in Table IX the activity of oxine is considerably reduced in 20 per cent blood medium. This was not due to the serum or to lysed red cells, since only intact red blood corpuscles were effective.

TABLE IX.—*Reversal of Action of Oxine by Blood.*

Basal medium : Beef extract, peptone water, pH 7·3. *Organism :* *Staph. aureus.* *Incubation time :* 48 hours at 37° C.

Medium.	Highest dilution of oxine inhibiting visible growth 1/M.	Comment.
Basal medium . . .	100,000	. .
+ 20 per cent horse blood .	6,400	. Marked reduction.
+ 20 per cent human blood A_2	3,200	. ,, ,,
+ 20 per cent lysed horse blood (freezing and thawing)	100,000	. No change.
+ 20 per cent horse serum .	50,000	. No significant change.

In an attempt to discover how red blood cells interfered with oxine action some preliminary experiments have been conducted, but without throwing any further light on this reversal. For instance, it has not been possible to show that intact red blood corpuscles remove oxine or metallic ions from a medium or lower its Eh. The need for further work on this point is clearly indicated.

DISCUSSION.

The results presented here confirm that the action of oxine depends upon its property of chelation, i.e. of forming non-ionized complexes with the ions of heavy metals, as was anticipated in Part III. At the commencement of this work it was thought that this chelation injured the bacterium by removing an essential trace metal from an important cellular enzyme-system. Fresh evidence in support of this conception has come to light. But this is not the complete picture of the action of oxine on Gram-positive organisms and on the genus *Neisseria* ; evidence is brought forward here that, for these bacteria, an additional and no less important cause of cell-injury is another chelation phenomenon, namely the formation of a toxic complex between oxine and the iron or copper ions of the medium. The lethal effect of the oxine in association with these metals appears to be directly due to the induced oxidation of a susceptible group

(e.g. an –SH group) associated with a vital metabolic process. Some of the evidence suggests that this group is normally protected by cobalt, and that the withdrawal of cobalt by chelation with oxine is a factor contributing to the death of the organism. With Gram-negative organisms (exclusive of *Neisseria*) no such metallo-toxic effect could be demonstrated, and death seems to be due entirely to metal-deprivation.

Before discussing the evidence leading to these conclusions, it is essential to consider quantitative aspects of the combination between oxine and metallic ions. The first reaction between oxine and a divalent kation is exemplified as follows :

$$C_9H_6N(OH) + Cu^{++} \rightleftharpoons C_9H_6N(OCu^{+}) \quad . \quad . \quad . \quad (i)$$

The products of this reaction are ionized and water-soluble. With the kations used in the present work (except in the cases of calcium and magnesium) the reaction does not stop at this stage, at or below pH 7, but another molecule of oxine becomes linked to the metal, as follows :

$$C_9H_6N(OCu^{+}) + C_9H_6N(OH) \rightleftharpoons (C_9H_6NO)_2Cu \quad . \quad . \quad . \quad (ii)$$

The products of this reaction are non-ionized and water-insoluble, but highly soluble in lipids. They are all stable to autoxidation except the ferrous complex, which rapidly oxidizes in air to the ferric complex $[(C_9H_6NO)_3Fe]$, which is green and not so liposoluble as the red ferrous complex.

The reactions depicted in both equations (i) and (ii) are instantaneous and require no activation energy. They are completely reversible, but the equilibria lie far to the right except for Mg^{++} and Ca^{++}. The equilibrium constants for equation (i) have been determined for these studies by potentiometric titration (with 0·1 N-potassium hydroxide) of an acidified solution of oxine (0·004-M) to which the appropriate metallic salt (0·002-M) had been added. The constants for equation (ii) could not be obtained in this way, because precipitates always formed at the higher pH values encountered and this led to a state of disequilibrium. However, the overall constant for both equations (i.e. the constant with which the biological results are apparently concerned) has been calculated on

TABLE X.—*Comparative Avidity of Oxine for Metallic Kations.*

Kation.	Logarithm of overall stability constant.*
Cu^{++}	23·4
Ni^{++}	18·7
Co^{++}	17·2
Fe^{++}	15·0
Cd^{++}	13·4
Mn^{++}	12·6
Mg^{++}	(4·5)†
Fe^{+++}	34·0

* Calculated as (2 log K′)−1 from the first-stability constant (K′) which is, e.g.

$$\frac{[C_9H_6N\,(OCu^{+})]}{[\text{oxine anions}]\,[\text{free metallic ions}]}$$

† First-stability constant only. At pH 7·3 only one out of three magnesium ions are attached to a molecule of oxine and no magnesium ion is attached to two molecules. Calcium is even more feebly chelated.

the basis that the second constant is one-tenth of the first one. This figure for the relationship between the two constants is derived from titrations where the complexes remain in solution throughout, viz. (*a*) titrations of oxine in aqueous dioxane (Maley and Mellor, 1949) and (*b*) titrations of oxine sulphonic acid in water. The overall constants calculated in this way are given in Table X. It was not possible to include zinc, because of solubility difficulties, but it would appear from the work of Maley and Mellor that its position falls between cobalt and nickel.

Oxine has a powerful antibacterial action against all Gram-positive organisms (including *Mycobacteria* and *Clostridia*, not shown in Table I), and also against *Neisseria*. The action on these oxine-sensitive types is specifically prevented by adding traces of a cobaltous salt to the medium. In contrast, oxine has in general only a weak antibacterial action against Gram-negative rods and this action is antagonized not by a single metallic ion, but by a number of ions, particularly iron, zinc and copper (cf. Table I). This multiple metal antagonism of Gram-negative rods is difficult to study, because the high concentrations of oxine necessary for inhibition of growth necessitate high concentrations of metallic salts, and this results in the precipitation of metal-oxine complexes. Hence we have given more attention to the quantitatively reproducible protection of oxine-sensitive organisms by cobalt. At first we were inclined to believe that the added cobalt functioned merely by combining with the oxine, and thus preventing it from exerting its normal biological action. However, reference to Table X shows that oxine has 30 times (i.e. antilog 1·5) the affinity for nickel that it has for cobalt. From the results given in Table VI it is evident that the nickel-oxine complex is non-toxic. Hence if it were merely a matter of inactivating the oxine, nickel would be 30 times as efficient an antagonist as cobalt.

The fact that cobalt is uniquely able to effect antagonism (Fig. 1) suggests that cobalt may be playing an important part in the metabolism of all Gram-positive organisms and of the *Neisseria*. It is true that these bacteria will multiply freely in a medium in which cobalt cannot be detected by spark spectrography. But the levels at which cobalt is biologically active in other systems falls far below the limit of detection by this means (about 0·2 part per million). Although Fildes and Richardson (1937) were able to grow *Staph. aureus* in a synthetic medium containing no *intentionally added* cobalt, this element would almost certainly have been added in the ferric ammonium sulphate (M/8000), and probably in the other salts (M/100) and in the amino-acids which were mainly of natural origin. (A substance that is 99·99 per cent pure, e.g. a salt of analytical reagent purity, has 6×10^{17} foreign molecules in each gramme, taking the average M.W. as 100.) Further, no growth stimulation of *Staph. aureus* by added cobalt can be demonstrated (Gibson, unpublished), which is not surprising in view of the above facts, because even our oxine-treated medium must contain many times the concentration of cobalt required for maximal growth. The cobalt potentiation of penicillin reported by Strait, Dufrenoy, Pratt and Lamb (1948) was considered by them to be due to the growth-stimulating effect of trace amounts of added cobalt on their organism (*Staph. aureus*).

The suggestion that cobalt may play a vital metabolic role in many Gram-positive bacteria is less surprising to-day than in 1946, when we discovered these cobalt-reversals. It is now known that vitamin B_{12} (which contains 4·5 per cent of cobalt) is essential for the aerobic growth of *Lactobacillus leichmanii* and

Lactobacillus lactis Dorner, and both these organisms are commonly used for the determination of this vitamin. Further, B_{12} has been isolated from three other Gram-positive bacteria, viz. *Mycobacterium smegmatis*, *Bacillus subtilis* and *Lactobacillus arabinosus* (Rickes, Brink, Koniuszy, Wood and Folkers, 1948).

Although the withdrawal of cobalt appears to be an important stage in the toxic action of oxine on Gram-positive bacteria, the toxic complexes formed by oxine with other trace metals present in the media are of at least equal importance. This was simply and unequivocally demonstrated by repeatedly treating a broth medium with oxine and extracting the metal-oxine complexes so formed with chloroform (see Experimental Section). After four such treatments the anti-bacterial activity of oxine, when tested in this oxine-extracted medium, began to decrease, until after 10 treatments oxine was no longer antibacterial in the medium (*cf.* Table IV). Chloroform-extraction in the absence of oxine did not change the antibacterial titre of oxine. Evidently oxine plus a lipoidal solvent have together removed a substance in whose absence oxine cannot exert its toxic action. Such a substance is obviously a metallic ion, because these alone are converted by oxine to chloroform-soluble complexes. It was soon found, in the case of *Staphylococcus aureus* (Table VII), that the addition of copper and iron ions in traces (about 0·3 part per million) restored the bacteriostatic activity of oxine to a level equal to that found in the untreated medium (such amounts of these ions were harmless in the absence of oxine). It was obvious from the blackening of the chloroform phase that considerable amounts of the iron-oxine complexes were removed in the first three treatments (all oxine complexes are yellow except those of Fe^{++} and Fe^{+++}, which are red and green respectively, a mixture of the two being black). However, it required 10 treatments to elicit maximal lowering of end-point, at which stage the concentration of the effective ion had evidently reached a threshold value. The toxic effect of oxine + iron (or copper) in oxine-treated medium is prevented by cobalt.

That oxine markedly inhibited oxygen uptake by *B. subtilis* in a glucose-yeast extract medium but not in glucose (Keilin and Hartree, 1947) can now be explained on the lines that the yeast extract supplied the iron required to potentiate the activity of oxine.

The likely mode of action of the toxic oxine-metal complexes is illuminated by the experiments in which the oxidation-reduction potential of the medium was lowered by the addition of ascorbic acid (Table VIII). A potential below − 0·06 volt (as measured by the decolorization of dyes) permitted bacterial growth in sixteen times the normal inhibitory concentration of oxine.

The overall picture of the toxic action of oxine, as furnished by these results, is that the iron or copper oxine complex acts as a catalyst which promotes the destructive oxidation of a group (e.g. an – SH group) on a metabolite or enzyme that is vital to the affected cells. This toxic action is preventable by the addition of traces of cobalt to the medium: It is possible that this protective action is actually the replacement of cobalt (removed by oxine), which normally protects this oxidizable group. This conception is supported by the fact that the toxic action can also be prevented by lowering the oxidation-reduction potential, thus either preventing this destructive oxidation, or else bringing into play a new metabolic pathway which circumvents it.

It remains to be shown whether it is the copper or the iron of common media which is the more important in making oxine behave as a powerful antibacterial.

This cannot be decided upon the evidence presented here, but results to be presented subsequently (in particular the lack of bactericidal action in concentrated, as opposed to dilute solutions of oxine) favour the greater importance of iron.

That the ferrous-oxine complex may not itself be the final toxic agent should be kept in mind. It can be seen from Table X that this complex is 150 times less firmly bound than the cobalt complex, and hence exists in equilibrium with a relatively large proportion of free (ferrous) ions, which are thus made available inside the organism in a higher concentration than may otherwise have reached there. In this connection it should be borne in mind that the iron-oxine complex is liposoluble, and hence it should penetrate the cell-membrane much more readily than free ferrous ions can. Moreover, two close structural analogues of oxine, viz. oxine-5-sulphonic acid and 4-hydroxypteridine, are not in the least antibacterial, and this inactivity may be correlated with the experimental findings that although they strongly bind the same metals as oxine does, the resultant complexes are not liposoluble.

One curious feature of this work is that *C. diphtheriae*, one of the Gram-positive organisms insensitive to oxine in oxine-treated media (Table V), can actually be protected from oxine by the addition of ferrous ions (Table I). It is likely that cupric ions are the co-toxicants in this case, but it cannot be excluded that metals not examined in this paper may have a special relevance for particular organisms.

Likewise, in view of the widespread occurrence and importance of various heavy metals in the economy of living cells, it is likely that oxine damages other metabolic processes than those discussed here. For example, it has recently been shown that the uptake of glutamic acid by *Staph. aureus* is prevented by lower concentrations of oxine than are needed to produce the antibacterial effects used as a criterion in the present work. This inhibition of glutamic acid assimilation was traced to a manganese deficiency induced by the chelating action of oxine (Gale and Mitchell, 1949).

SUMMARY.

The antibacterial activity of oxine (8-hydroxyquinoline) has been further investigated. The following are the principal findings:

(*a*) Cobalt is unique among metallic kations in antagonizing the action of oxine against Gram-positive bacteria and the *Neisseria*, whereas several metals (notably iron, zinc and copper) can antagonize its action against Gram-negative rods (Table I).

(*b*) Oxine is much less antibacterial to Gram-positive species in media from which trace metals have been removed below a critical concentration (Table V). In the cases of *Strept. pyogenes* and *Staph. aureus* it is no longer antibacterial at all.

(*c*) In such media the activity against *Staph. aureus* is restored by the addition of traces of iron, copper or cadmium salts (Tables VI and VII).

(*d*) By lowering the oxidation-reduction potential below $-0{\cdot}06$ volt at pH 7·0, the antibacterial action of oxine is prevented (cf. Fig. 3).

(*e*) The presence of intact red cells in ordinary media lowers the inhibitory end-points (Table IX).

In the case of *Staph. aureus*, which has been the most completely explored, the following conclusions have been drawn :

(*a*) Oxine *per se* has no antibacterial action, and is toxic only because of the iron and copper ions present in the medium. The toxic effect is undoubtedly initiated by chelation (i.e. formation of a toxic metal-oxine complex), and is completed by a subsequent poisoning effect of the metal, either in combination with oxine or as free ferrous ions liberated after transport through the cell-wall in the chelated condition.

(*b*) This poisoning apparently takes effect through catalysis of the oxidation of an essential enzyme or metabolite. This destructive oxidation can be prevented by lowering the oxidation-reduction potential (Table VIII).

(*c*) The inhibition of the antibacterial action of oxine by cobalt is not due to the high stability and low toxicity of the cobalt-oxine complex, because the nickel-oxine complex is 30 times as stable (Table X) and equally non-toxic, yet it completely lacks the antagonizing powers of cobalt (see Fig. 1). This result suggests the possibility of an essential role for cobalt in all Gram-positive organisms (and in the *Neisseria*), e.g. as a protector of groups susceptible to destructive oxidation.

The overall conclusion drawn from these studies is that oxine acts against bacteria by means of chelation. The chelation of different metals is critical for different classes of organisms and for different functions in the one organism.

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THE ROLE OF BACTERIAL ANTIGENS IN DIPHTHERIA PROPHYLACTICS.

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THE protective agent in diphtheria immunity is most believed to be solely antitoxin, since attempts to protect by the use of bacterial vaccines have always been unsuccessful (Debré, Ramon and Thiroloix, 1931; Ørskov, Krag Andersen and Poulsen, 1944) and protection against the disease caused by types *gravis*, *intermedius* or *mitis* can be achieved by immunization with toxoid obtained from culture filtrates of strain P.W.8 (Parish, Whatley and O'Brien, 1932; Povitsky, Eisner and Jackson, 1933; Ørskov *et al.*, 1944) classified as a *mitis* form by Tarnowski (1942*b*).

However, the specific part of the proteins present in toxoid in the form in which it has hitherto exclusively been used in such experiments as those cited above, amounts to only 1/4 to 1/3 of the total protein present, the rest being soluble non-toxin proteins presumably originating from the bacterial bodies.

The belief that bacterial antibodies may play a role in protection against diphtheria is sometimes met with in the literature, especially where the effect of serum treatment is discussed (Park, Williams and Mann, 1922; Huang, 1942). The latter describes the formation of specific agglutinins in diphtheria patients during the convalescent stages and has put forward the hypothesis that "the absence of bacteraemia and the important part the toxin plays in the causation of the disease may mask the differences due to antibacterial property."

The purpose of the present work, therefore, is a closer experimental analysis of the relative merits of a pure antibacterial and a pure antitoxic immunity, or a combination of both, in the dynamics of an incipient infection.

This required a pure bacterial and a pure toxin- or toxoid antigen, as well as a technique for infecting animals, that would permit of circumstances closely simulating the human pathology of the disease.

The two latter essentials have only recently become available, the first of these through the isolation of pure diphtheria toxin or toxoid free from other protein fractions (Eaton, 1936; Pappenheimer, 1937; and Pillemer, Wittler, Clapp and Adam, 1948), and the second through a technique developed by Ørskov (1948), by which a pathological picture in guinea-pigs very similar to that seen in man is achieved. This cannot be obtained as a pharyngeal infection, but can be produced by establishing a cutaneous focus of infection in the following way: A small area of skin on the ventral wall of the animal is shaved, and very gently scarified, care being taken to avoid bleeding. A small quantity of a virulent culture in broth is rubbed on the scarified zone, and the focus of infection covered with a piece of gauze moistened in saline and held in position with adhesive tape. This is followed by the development of a typical diphtheritic

membrane, with gross local oedema and death in 2 to 5 days. The post-mortem findings are those typical of diphtheria intoxication ; in no case have diphtheria bacilli been isolated from any of the organs.

REAGENTS AND TECHNIQUE.

Pure diphtheria toxoid.

Highly purified diphtheria toxoid, having a purity of 2100 Lf/mg. protein nitrogen and electrophoretically homogeneous, was adsorbed onto aluminium hydroxide gel to give a concentration of 1 mg. of aluminium and 50 Lf per ml. of the final suspension.

Bacterial vaccine.

C. diphtheriae gravis, Strain No. 1282, was used to prepare the vaccine. The microbe was grown on ascites agar (48 hours, incubation) to which had been added some ferrous sulphate to reduce toxin production to a minimum. The microbes were killed by suspension in formol-saline.

Soluble non-toxin culture filtrate protein.

This was prepared by growing strain P.W.8 for eight days at 34° C. on a medium (Pope and Llewellyn-Smith, 1932) to which had been added a gross excess of iron (Lawrence and Pappenheimer, 1948). The culture filtrate was concentrated and freed from non-protein material by ultra-filtration. The final product contained 0·69 mg. protein nitrogen and about 0·05 unit of toxin per ml. which accounted for approximately 0·003 per cent of the total protein present. After being detoxified with formalin, $Al(OH)_3$—gel was added to give a concentration of 1 mg. of aluminium per ml. of suspension.

Material of infection.

The microbes used for this purpose were of the same strain as used for the bacterial vaccine, but grown for 20 hours only on the ascites-agar, without the addition of ferrous sulphate, and then suspended in nutrient broth.

The technique of infection.

This has been described above.

Serum agglutination.

Titrations were made, using the same strain as that used for the vaccine as antigen. The preparation of the antigen and the titration technique were as described by Tarnowski (1942*a*), and the titres expressed as the highest dilution which produced detectable agglutination.

Antitoxin titres.

Antitoxin titres were determined by the intracutaneous method of Jensen (1933).

EXPERIMENTAL.

Part I.

One group of seven guinea-pigs was inoculated twice subcutaneously with 0·2 ml. of the adsorbed pure diphtheria toxoid, at a four weeks interval.

A second group received, in addition to the adsorbed toxoid, the bacterial vaccine. The latter was given in 8 injections intraperitoneally at 3 to 4 days' intervals, commencing with a dosage of about 12 millions, increasing to a maximum dosage of about 100 millions.

A third group received the course of inoculations with the vaccine only.

One week after the second injection of adsorbed toxoid, and four days after the last dose of vaccine, all animals were bled by cardiac puncture, and the antitoxin and agglutination titres of each serum measured.

The following day each animal was infected as described. The dose of living microbes chosen was ten times that number, which by previous trial resulted in nearly 100 per cent of deaths in normal animals; this latter was 10^5 organisms as estimated by live count.

As controls, a number of untreated pigs were infected with 10^5 and 10^6 organisms in the same manner.

From the results, which are given in Table I, it will be seen that a pure antitoxic response was obtained in Group 1, a pure antibacterial one in Group 3, and

TABLE I.—*Effect of Different Prophylactic Methods on the Survival of Guinea-Pigs Infected with Virulent* C. diphtheriae. Part I.

Antigens.	Guinea-pig No.	Antitoxin units per ml. at time of infection.	Agglutination titre at time of infection.	Observations after infection with *C. diphtheriae* type *gravis* 1282.
Pure diphtheria toxoid adsorbed on to $Al(OH)_3$.	8779	0·04	<1 : 25	Survived without symptoms.
	8780	0·65	,,	,, ,, ,,
	8781	5·0	,,	,, ,, ,,
	8782	5·0	,,	,, ,, ,,
	8783	1·0	,,	,, ,, ,,
	8784	2·2	,,	,, ,, ,,
	8785	5·5	,,	,, ,, ,,
Pure diphtheria toxoid adsorbed on to $Al(OH)_3$ + bacterial vaccine	8786	0·8	1 : 200	Survived without symptoms.
	8787	4·0	1 : 800	,, ,, ,,
	8788	12·5	1 : 1600	,, ,, ,,
	8789	1·6	1 : 400	,, ,, ,,
	8790	2·0	1 : 400	,, ,, ,,
	8791	5·0	1 : 1600	,, ,, ,,
	8792	0·03	1 : 1600	,, ,, ,,
Bacterial vaccine	8793	<0·001	1 : 800	Died 3rd day.
	8794	,,	1 : 1600	Ill 3rd–5th day, survived.
	8795	,,	1 : 400	Died 3rd day.
	8796	,,	1 : 800	,, 5th ,,
	8797	,,	1 : 800	,, 3rd ,,
	8798	,,	No titrat.	Ill 3rd–5th day, survived.
	8799	,,	1 : 100	Died 3rd day.
Controls	8804			Died 2nd day.
Infecting dose 10^5 organisms	8805			,, 5th ,,
	8806			,, 2nd ,,
Controls	8801			Died 5th day.
Infecting dose 10^6 organisms	8802			,, 2nd ,,
	8803			,, 3rd ,,

a combined response in Group 2. It can be seen also, that only those animals which possessed antitoxin survived the infection without any symptoms of the disease. No differences in either local or general reaction were noticed between those which received toxoid alone, and those which received both toxoid and massive vaccine inoculations. The presence of the gauze and adhesive tape prevented daily observation of the infection sites, but a few animals from each of these two groups were killed at the seventh day and autopsied. None showed any signs of general reaction, and only vestiges of a slight local reaction. No microbes could be isolated from the site of infection.

In the group immunized with vaccine only, five out of seven died within the same time intervals as the controls. Post-mortem examination of all of these five showed the same pathological changes as the controls—diphtheritic membrane oedema, adhesions and local haemorrhages at the site of infection, and in every case *C. diphtheriae* was isolated. The usual picture of diphtherial intoxication was found at laparotomy—red and enlarged adrenals and pleural exudation.

The surviving two animals from this group were examined on the 15th day. They both showed a necrotic area 1 to 2 cm. in diameter at the infection site, otherwise nothing abnormal.

The survival of these two animals seemed to indicate a certain protective effect from the bacterial antibodies present at the time of infection since no antitoxin was detectable in their sera (Table I).

Part II.

In respect of the first experiment it was felt that the animals had been excessively immunized against toxin, and that therefore, if the same dosage of vaccine was used, and much less toxoid, differences due to the antibacterial component of the total immunity would show themselves more clearly. It was thought necessary also to examine the effect of the non-toxin proteins derived from the P.W.8 culture filtrates since these are found in almost all prophylactics hitherto used in human immunization, in experimental work using guinea-pigs, as well as in the production of therapeutic sera in horses.

The second series of experiments was divided into five parts :

Group A were given *one injection only of* 1 *Lf* of the adsorbed toxoid.

Group B were also given 1 Lf dose of adsorbed toxoid supplemented with the full course of bacterial vaccine inoculations.

Group C were given, in addition to the 1 Lf of adsorbed toxoid, three subcutaneous injections of the adsorbed soluble non-toxin protein from P.W.8 culture filtrates (see Reagents) at 2-weekly intervals.

Group D received the vaccine injections only.

Group E received the three injections of adsorbed soluble non-toxin protein only.

Group F control groups.

Each group comprised 20 animals. The inoculations were arranged so that bleedings were made four weeks after the injection of toxoid, and seven days after the final injection of vaccine and adsorbed non-toxin culture filtrate proteins. The animals were infected, as before, with 10^6 organisms 24 hours after the bleedings. As in the first experiment, antitoxin and agglutination titres were measured individually. In Table II where the results are summarized, these

TABLE II.—*Effect of Different Prophylactic Methods on the Survival of Guinea-pigs Infected with Virulent* C. diphtheriae. Part II.

Group.	Antigens.	Antitoxin units per ml. at time of infection (Geometric mean).	Agglutination titre at time of infection (Geometric mean).	Death rate after infection.	Antitoxin units per ml. 7 days after infection (Geometric mean).
A	Pure toxoid	0·052	<1 : 25	0/20 = 0%	1·08
B	Pure toxoid + vaccine	0·030	1 : 400	0/19* = 0%	0·44
C	Pure toxoid + soluble bacterial protein	0·40	1 : 80	1/17* = 6%	1·07
D	Vaccine	<0·001	1 : 200	5/17* = 29%	<0·001
E	Soluble bacterial protein	<0·001	<1 : 30	8/20 = 40%	<0·001
F	Controls				
	Infecting dose 10^5	<0·001	<1 : 25	0/10 = 0%	<0·001
	„ „ 10^6	<0·001	„	3/10 = 30%	<0·001

* In these groups a total of seven animals died from intercurrent infection, or as a result of the bleedings.

titres are given as the geometric mean for each group. It will be noticed that in this experiment the infecting dose (10^6) of microbes killed only 30 per cent of the controls, and 10^5 microbes caused no deaths or reactions in another group. Although this was unintentional, it clearly was an advantage in respect of the problem under study, as it means we were comparing the advantages of different prophylactic procedures at a D.L. 30 level—a very sensitive one for such work.

As in the first experiment, immunization with diphtheria toxoid gave a pure antitoxic response and the bacterial suspension a pure antibacterial one.

The inoculation of the non-toxin soluble protein led to no measurable antitoxin production in the group receiving this antigen only (Group E), the very small amount of toxoid present evidently being insufficient to sensitize the antitoxin producing mechanism. That it had been adequate to act as a secondary stimulus may, however, be seen from the Group C figures where the mean antitoxin titre prior to infection was significantly higher than the means of Groups A or B.

The non toxin soluble protein gave rise to a slight production of agglutinins towards the *gravis* strain. No precipitin tests with the non-toxin protein as antigen were undertaken since, by mistake, all of this antigen had been adsorbed on to the $Al(OH)_3$.

It will be seen from Table II that only those animals which were immunized with toxoid acquired increased resistance towards the infection.

A comparison of the death rates among those groups which received toxoid Groups A, B and C, with those which did not, D and E, gives 1 in 56, or 1·8 per cent as against 13 in 37 or 33 per cent (with 30 per cent for the controls). The difference between these rates is highly significant, P being <1 : 1000.

It should be put on record that the solitary animal which received toxoid and died (Group C) was found to possess <1/1000 U. of antitoxin per ml. of serum at the time of infection.

The post-mortem findings on all the animals which died were as before, and no differences were discoverable between those which had received vaccine or soluble non-toxin protein, and the controls.

No correlation was found between the agglutination titres and survival; for example, in Group D, where the homologous strain was used as the vaccine all the animals which died had a titre of >1/200, whereas five out of seven of the surviving animals had <1/100.

Again, of the 20 sera from those animals which had received the soluble non-toxin protein from a heterologous strain (P.W.8, 13 to some extent agglutinated the *gravis* strain used for infection, whereas 7 contained no measurable agglutinin; of the latter group, 2 animals died (30 per cent), and of the former 6 (46 per cent).

The individual behaviour of those animals in the toxoid groups which failed to make detectable antitoxin as a result of their prophylactic inoculation, is of no small interest, and is detailed in Table III. It is here clearly demonstrated

TABLE III.—*Responses of those Animals Previously Inoculated with Toxoid, yet Failed to make Detectable Antitoxin (Table II, A, B and C) to a Subsequent Infection with* C. diphtheriae.

Group.	Guinea-pig No.	Antitoxin units per ml. at time of infection.	Agglutination titre at time of infection.	Observations after infection.	Antitoxin units per ml. 7 days after infection.
A	6041	<0·001	1 : 50	Survived without symptoms of disease	1·0
	6046	,,	<1 : 25	Do.	1·4
	6053	,,	1 : 25	,,	0·16
	6056	,,	<1 : 25	,,	1·0
B	6061	,,	1 : 400	,,	0·7
	6065	,,	1 : 200	,,	0·16
	6069	,,	1 : 800	,,	1·6
	6075	,,	1 : 400	,,	<0·001
C	6008	,,	<1 : 25	Died 3rd day	

that these animals which seemed to be in the same state of non-antitoxic immunity as the animals in Groups D and E (Table II) really did have a basal immunity, which enabled them to produce rather amazing amounts of antitoxin in the course of 7 days as a result of the toxin produced at the site of infection. In contrast to this, all the surviving animals in Groups D and E still possessed no detectable antitoxin in the same time interval after infection (see Table II).

This immunological difference is reflected numerically in the death rates, which are 11 per cent among the animals in Table III as against 35 per cent in Groups D + E (Table II). These death rates, it is true, do not differ significantly, but bearing in mind the few animals under review in Table III and the low death rate, a significant difference was not to be expected. These findings may, therefore, be considered as additional proof of the pre-eminent rôle of antitoxic immunity in resistance towards diphtherial infections.

DISCUSSION.

It has long been believed and was recently proved by Pappenheimer, Lawrence and Sherwood (1948) that the untoward reactions met with in diphtheria immunization, and which especially among adults, may be rather severe, are partly caused by sensitivity towards non-toxin diphtherial protein in the prophylactics. It would, therefore, from this point of view be desirable to exclude such material from prophylactics.

The above findings seem to prove that only that part of the protein which accounts for the pure antitoxin producing antigen is of value in diphtheria prophylactics, as immunization with neither the non-toxin proteins nor with homologous bacteria increased the resistance towards infection, and had no protective value by themselves.

Prophylactics prepared from pure or highly purified toxoids which, as a result of the work of Pillemer *et al.* (1948) and Holt (1947) can now be made on a large scale, may therefore be used with every confidence. It is to be expected also that the removal of so much diphtherial non-toxin protein, as is the case in these prophylactics, will result in a marked reduction in the incidence of reactions in mass immunization. At the same time adsorption onto mineral carriers may be facilitated by the exclusion of useless competing protein.

Furthermore, the findings enable us to rely on antitoxin titres as a reliable index of specific immunity towards diphtheria.

SUMMARY.

Using Ørskov's technique, no difference in resistance towards infection with virulent *C. diphtheriae* (*gravis*) could be demonstrated between guinea-pigs immunized with pure toxoid only, and those which received in addition several injections of either a vaccine of the homologous strain, or of the soluble non-toxin proteins from a filtered broth culture of the Park William 8 Strain, from which the toxoid was made.

Immunization with neither of the latter antigens led to any protection against the experimental infection.

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